CATECHOLAMINERGIC INNERVATION OF THE CAT SPINAL CORD.

by

Christopher Alfred Doyle

Doctor of Philosophy

University of Edinburgh

1994

DECLARATION.

I certify that the authorship of this thesis is entirely my own and that the work presented in it is substantially my own. The one exception is the horseradish peroxidase implantation technique, which was performed by Dr. D.J. Maxwell.

Some of this material has been published in collaboration with Dr. D.J. Maxwell, and reprints are included in the thesis as an appendix. None of this work has been submitted for any other degree or professional qualification.

C.A. Doyle.

Contents.

FOR

HEATHER

I thought I heard the sound of my name,
As the warmth of your arms wrapped around me,
In a naked dance we drifted the plains,
- A daydream that you came and found me.

A songbird sings and my heart it rings,
This moment all frozen and gleaming,
You looking breathless consuming my mind,
Just me and my crazy dreaming.

She's somewhere out there with my heart in her care,
And our dreams in the breezes that caught them,
All alone now on this apple tree down,
Amid the showering yellows of autumn.

Acknowledgements.

My sincere thanks go to Dr. David Maxwell for his invaluable assistance and advice throughout the course of this work. Many thanks are also due to Professor Alan Brown for his interest and support in the project.

A very special thankyou is owed to Heather Anderson for expert histological and photographic assistance, in addition to her friendship, love & constant encouragement throughout the last four years. The light microscopic drawings were skillfully reproduced by Colin Warwick.

The antiserum to tyrosine hydroxylase was a generous gift from Dr. John Powell (University of Oxford) to Dr. Cali Ingham, who kindly allowed me to use it in these studies. The project was funded by a Medical Research Council programme grant awarded to Dr. David Maxwell and Professor Alan Brown.

Finally, I would like to acknowledge the support that my parents and family and friends have given to me during the course of this work. In particular I would like to thank Peggie and Esther Binnie for all of their love and for making me smile and, to Esther, I extend my warmest and deepest affection for a beautiful weekend in September in which we toured the graveyards of Edinburgh and for many cherished moments since. I would also like to mention Ian Van Hek, Fran and Rob Bryan, Colin Anderson and Alasdair Olufson, who died in 1991, before completing his PhD.

Abstract.

The organization of catecholamine (CA)-containing nerve terminals in the cat spinal dorsal horn was examined in an immunocytochemical study employing antisera against tyrosine hydroxylase and dopamine-ß-hydroxylase. Light microscopic analysis revealed that varicose axons were concentrated in laminae I, II & IV. Correlated ultrastructural analysis showed that these terminals usually formed single synapses with dendrites or somata, but not with other axon terminals. This suggests that descending catecholaminergic axons regulate sensory transmission through the dorsal horn via a postsynaptic action upon dorsal horn neurons.

Using the retrograde tracer horseradish peroxidase to label particular groups of dorsal horn neurons, it was shown that the postsynaptic dorsal column (PSDC) pathway is a major projection target of CA-containing axons. Over 60% of these cells were found to have dopamine-B-hydroxylase immunostained varicosities closely apposed to their somata and/or proximal dendrites, and correlated electron microscopic analysis confirmed that many of these contacts were regions of synaptic association. In contrast, the cells of the spinocervical tract (SCT) did not receive a major innervation from these axons when viewed with the light microscope.

The lateral cervical nucleus (LCN), the termination site of SCT cells, was found to possess a dense innervation from CA-containing axons. These fibres were

present throughout the nucleus and synapsed with dendrites and somata, including those of large cells in the lateral region, but not with other axon terminals. This suggests that catecholaminergic axons in the LCN regulate the activity of LCN neurons but not the terminals of SCT cells.

It has been suggested that many catecholaminergic axons in the dorsal horn may contain neuropeptide Y (NPY), and an examination was made of NPY-immunoreactive axons to test this hypothesis. Light microscopy revealed a heavy concentration of NPY-positive profiles in laminae I-II but only low to moderate numbers in III-VI. Fine structural examination showed most of these profiles to be axon terminals but a few structures were observed which may have been NPY vesicle-containing dendrites. The postsynaptic targets of NPY terminals were mostly dendrites or somata, but many (27%) formed axo-axonic synaptic junctions. In laminae I-III, the postsynaptic axon terminals were sometimes the central boutons of glomeruli. These findings suggest that NPY axons regulate sensory transmission by a postsynaptic action upon dorsal horn neurons and a presynaptic action upon primary afferent terminals. NPY boutons often formed more than one synapse and some of these arrangements were confirmed to be triads. Clearly, NPY profiles are organized in a much more complex way than those containing CA, which suggests that co-localization between the two neurotransmitters in the spinal dorsal horn is, at best, limited.

GENERAL INTRODUCTION.

The results presented in this thesis are concerned primarily with the ultrastructural organization of catecholamine-containing nerve terminals within the spinal cord (particularly the dorsal horn), and the effects that these structures are likely to have on sensory transmission to the brain. An examination was also made of axons containing neuropeptide Y to assess whether co-storage between catecholamines and this peptide is likely in the spinal cord.

In order to introduce the results correctly it is necessary to give an account of the organization of sensory pathways in the dorsal horn, with particular attention being given to the origin, distribution and actions of axons containing catecholamines and neuropeptide Y.

(1). ORGANIZATION OF THE DORSAL HORN.

(a). **Introduction.**

The dorsal horn of the spinal cord is the place where information from the body surface and underlying tissues reaches the central nervous system. It is here that the first stage in the integration of sensory messages takes place, where the ascending pathways to the brain originate and where the brain can exert control over the messages through a variety of descending pathways. The

spinal dorsal horn is defined as that part of the grey matter which lies dorsal to the central canal. Rexed (1952) divided the the dorsal horn into six layers or laminae based upon the cytoarchitectonics of the region as viewed in Nissl stained sections of cat spinal cord (see Figure 17), that is, the scheme is based purely upon the size, shape and density of somata. Despite this shortcoming, however, the scheme has proved successful and inputs to the dorsal horn from primary afferent axons often respect the cytoarchitectonic boundaries, as do a number of the dendritic trees of dorsal horn neurons.

(b). **Primary afferent neurons.**

(i). STRUCTURE AND FUNCTION OF PRIMARY AFFERENT NEURONS.

Primary afferent neurons (sensory neurons) are located in the dorsal root ganglia and have a peripheral process that receives information from sensory receptor organs and a central process that transmits this information centrally (Lieberman, 1976). The central processes are often referred to as dorsal root axons because they travel in the dorsal root on their way to the spinal cord. It has been reported that the axons in feline dorsal roots segregate into large and small fibres as they enter the cord (Ranson, 1914). Large axons are myelinated, whereas small axons can be either myelinated or unmyelinated. It is generally assumed that the diameter of the fibres is related to the size of their cell bodies in the dorsal root ganglia, which can be

divided into two groups (large, pale (A) cells and small dark (B) cells), according to their morphology (Lieberman, 1976).

In cutaneous nerves, the largest myelinated axons belong to the Aαß class, whereas the small myelinated fibres belong to the Aδ group. Aαß fibres conduct impulses at 30-100m/s and Aδ fibres conduct at 4-30m/s (Boivie and Perl, 1975). Unmyelinated fibres (C fibres) conduct at less than 2.5m/s (Gasser, 1950). Visceral nerves share the terminology of cutaneous nerve axons (Mei, 1983). A different terminology is used for muscle and joint nerves. The myelinated afferent fibres of muscle nerves are subdivided into groups I, II and III (Lloyd and Chang, 1948). In cats these groups of axons conduct at 72-120, 24-71 and 6-23m/s respectively (Hunt, 1954). Muscle nerves also contain unmyelinated, or group IV, afferent fibres (Stacey, 1969). Joint nerves, which arise from Golgi tendon organs in ligaments or from muscle spindles, resemble muscle nerves (Andersen et al., 1967; Burgess and Clark, 1969).

Primary afferent neurons have also been characterized according to the various stimuli required to activate the sensory receptors associated with their peripherally located processes. Within the skin and underlying tissues three types of primary afferent neuron have been identified on this basis. Mechanoreceptors are most readily activated by mechanical changes. In the skin, several different kinds of mechanoreceptor have been

described, which respond to stimuli such as indentation of the skin (e.g. Slowly Adapting Type I & II receptors) or the movement of hairs (e.g. Guard or Tylotrich receptors) (Burgess and Perl, 1973). Noxious stimuli produce no greater excitation of these receptors than do innocuous ones. Most cutaneous mechanoreceptors conduct impulses in the Aαß range (Brown and Iggo, 1967; Burgess et al., 1968), but down hair (D) receptors are innervated by axons of the Aδ type (Brown and Iggo, 1967). Nociceptors are defined as sensory endings that respond to stimuli that threaten to damage or actually damage tissue. The studies of Perl's group, in the cat and monkey, (Bessou and Perl, 1969; Burgess and Perl, 1967, 1973; Perl, 1968) have demonstrated that cutaneous nociceptors are of two main types: Aδ mechanical nociceptors and C polymodal nociceptors. Aδ mechanical nociceptors are excited best by mechanical stimuli that damage the skin, and do not respond to noxious heat, extreme cold, or algesic chemicals. C polymodal nociceptors are an abundant receptor type, especially in primates, and they respond well to intense thermal and mechanical stimuli as well as to irritant chemicals. Similarly, muscular and articular nociceptors are associated with fine myelinated (type III) and unmyelinated (type IV) fibres (Paintal, 1960; Mense and Scmidt, 1974; Schaibel and Schmidt, 1983), and visceral nociceptors associated with C fibres have been described (Sugiura et al., 1989). Thermoreceptors signal innocuous changes in skin temperature. Two types have been

described, which respond to warming (warm receptors) and cooling (cold receptors) of the skin. These afferents conduct impulses in the Aδ and C ranges (Iggo, 1969; Iruichijima and Zotterman, 1960).

(ii). TERMINATION OF PRIMARY AFFERENT AXONS IN THE SPINAL DORSAL HORN.

Results from morphological studies have demonstrated that the branching pattern of primary afferent axons within the spinal cord is quite specific and varies according to the type of afferent unit. Golgi staining methods first pointed to the superficial laminae as the projection site of fine fibres and to the deeper laminae as the projection site of coarse fibres (Ramón Y Cajal, 1909; Pearson, 1952), and from early days laminae I and II have attracted attention with regard to to a role in nociception (Pearson, 1952).

A number of experimental approaches have been used to confirm these morphological findings. For instance, following section of the dorsal roots, degeneration can be observed at the ultrastructural level which is manifest in the different laminae after delays that relate to the fibre diameter; myelinated fibres degenerate before unmyelinated fibres. In the study of Ralston and Ralston (1979), which was restricted to laminae I-III of the monkey, degeneration first appeared 18 hours after rhizotomy in laminae IIi and III. A second phase of degeneration occured in laminae I and IIo

two days after rhizotomy, and a third phase of degeneration was observed in lamina II approximately 5 days later. These authors suggested that the three phases of degeneration corresponded to a loss of Aαß, Aδ and C fibres respectively. Using a similar approach, several authors have labelled primary afferent axon collaterals in several mammals (rat, cat and monkey) by applying horseradish peroxidase (HRP) to partial lesions of dorsal rootlets (Light and Perl, 1977, 1979a; Gobel et al., 1981). Application of HRP to a lesioned portion of the lateral division (where the fine fibres are located) produced labelling principally in laminae I and II, whilst HRP application to the thick fibres of the medial division produced labelling in laminae III and IV.

Although the majority of fine myelinated (Aδ) and unmyelinated (C) fibres belong to nociceptors (Burgess and Perl, 1973; Sugiura et al., 1986), a few respond specifically to innocuous stimulation (Brown and Iggo, 1967; Iggo, 1969; Sugiura et al., 1986). It is therefore not sufficient, in the context of nociception, to simply trace the terminations of fine fibres. The only unambiguous approach in identifying with certainty the termination sites of nociceptive and non-nociceptive afferents is to intracellularly label functionally identified fibres. Light and Perl (1979b) have succeeded in intra-axonally injecting cutaneous Aδ mechano-nociceptors with horseradish peroxidase (HRP). The terminal arbors of these units were found throughout lamina I and the adjacent part of lamina II, and many

units also had a branch that entered and arborized in lamina V. The intra-axonal labelling of cutaneous C fibres is extremely difficult due to their fine diameter. However, Alvarez et al. (1993) have successfully impaled and labelled two C fibres with HRP in monkeys; one of which was positively identified as a nociceptor. The terminals of this C nociceptor were distributed throughout laminae I and II. Sugiura et al. (1986) overcame the problems associated with intra-axonal labelling of C nociceptors by injecting *Phaseolus vulgaris* leucoagglutinin into their cell bodies in the dorsal root ganglia of guinea pigs. Most of these cells (4/5) arborized exclusively in laminae I and II, but one unit also had a few branches in laminae III and IV. Thus intracellular staining techniques seem to indicate overlap in the terminal zones of Aδ and C nociceptors. However, the zone of arborization is confined almost exclusively to the superficial dorsal horn. The larger myelinated (Aαß) cutaneous primary afferent fibres, that innervate low threshold mechanoreceptors, have been studied extensively by Brown's group in the cat. Although each type of afferent unit has a specific branching and bouton distribution pattern, all distribute their input to laminae III-VI: they do not give boutons to lamina I or to lamina II, except very occasionally to the innermost part of II (Brown, 1981). Furthermore, the thinner (Aδ) cutaneous axons innervating D hair follicle receptors (Light and Perl, 1979b) also arborize in the

same way as their thicker brethren that innervate G and T hair follicles. Cutaneous C mechanoceptors, however, terminate in the superficial laminae (Sugiura et al., 1986).

The dorsal horn also receives primary afferent inputs from muscle, joint and visceral nerves. Low threshold muscle afferents (groups Ia, Ib and II) distribute collaterals mainly to the deeper regions of the spinal grey matter (laminae VII and IX), although some terminals are found in the deep dorsal horn (laminae IV-VI) (Brown, 1981). Visceral C nociceptors, with somata in the celiac ganglion of the guinea-pig (Sugiura et al., 1989), unlike their cutaneous counterparts (Sugiura et al., 1986), have a rather scattered distribution, giving off collaterals in the superficial (I,II) and deep (IV,V) dorsal horn. Branches also terminate in lamina X (region adjacent to the central canal) and contralaterally. Craig et al. (1988) have shown that the termination sites of knee joint afferents in the cat are laminae I and V-VII. No evidence was found for projections into laminae II-IV.

(c). **The neurons of the dorsal horn.**

Several attempts have been made to develop a system for classifying dorsal horn neurons, but none of the approaches suggested to date is completely satisfactory. Dorsal horn neurons may be classified according to several criteria, including their response to natural stimuli (e.g. nociceptive and non-nociceptive neurons),

the destination of their axons (e.g. propiospinal and projection neurons) or their morphology and location within the dorsal horn. The first part of this section deals with the different morphological cell types which can be found in each of Rexed's laminae. In the second part, a classification system is presented for dorsal horn neurons, which is based upon the destination of their axons and their cutaneous afferent inputs. It should be noted, however, that there will be some degree of overlap in the content of each of these sections.

(i). MORPHOLOGICAL FEATURES OF DORSAL HORN NEURONS.

Lamina I.

The most conspicuous occupants of lamina I are the large marginal cells of Waldeyer, although there are numerous small neurons about which very little is known (Rèthelyi et al., 1989). The major dendrites of marginal cells are arranged horizontally in a discoid (Szentágothai, 1964) or oval (Woolf and Fitzgerald, 1983) fashion, and remain within lamina I.

Lamina II (the substantia gelatinosa).

The small neurons of lamina II were divided into limitrophes and central cells by Ramón Y Cajal (1909).

Limiting cells are larger than central cells and their cell bodies are located in the outer part of lamina II, particularly at the lamina I-II border. The dendrites of

these cells form a fan emanating from the cell body and passing ventrally through laminae II-IV. Gobel (1978) termed these neurons stalked cells, because of their short stalk-like spines.

Central cells are the common cells of the gelatinosa, and are found throughout it. The cell bodies are small and have little cytoplasm, and the primary dendrites of these cells usually arise from the dorsal and ventral poles of the cell. These dendrites branch repeatedly within lamina II and give rise to an extensive arbor which is oriented in the rostrocaudal direction. Gobel (1978) termed these neurons islet cells, because they occur in clusters or islands.

Lamina III.

The cells of lamina III are spindle-shaped and have relatively little cytoplasm. They resemble, but are slightly larger than, the central cells of lamina II (Ramón Y Cajal, 1909; Rexed, 1952). The dendritic arbors of lamina III cells were not distinguished from lamina II cells in earlier studies, except that the rostrocaudal extension of the dendrites was greater in lamina III cells (Ramón Y Cajal, 1909; Szentágothai, 1964; Schiebel and Schiebel, 1968). Latter-day Golgi studies have confirmed that the majority of lamina III neurons have arbors oriented rostrocaudally and confined to the lamina of origin, but a few cells have been observed which arborize dorsoventrally and have dendrites that penetrate lamina II (Maxwell, 1985).

An important advance in understanding the dendritic architecture of lamina III came from intracellular staining studies of projection neurons of the spinocervical tract (SCT) and postsynaptic dorsal column (PSDC) pathway in cats (Brown et al., 1977b; Brown and Fyffe, 1981). SCT cells have their dendrites oriented more in the longitudinal plane than in the transverse plane, and compare well with the depictions of lamina III neurons from earlier Golgi studies, and although they have many dorsal dendrites, these dendrites do not enter laminae I or II. In contrast, the dendrites of PSDC cells frequently enter laminae I and II and are not restricted mediolaterally. These cells are depicted as dorsoventrally oriented cylinders or cones (Brown, 1981). It is important to remember though, that these two cell types form a minority of the cells in lamina III, and that most cells in lamina III have not been identified.

Lamina IV.

The neurons of lamina IV are rather heterogenous; many small cells are present, but there are also a number of very large, prominant cells (Rexed, 1952). The large cells have long, spiny dendrites that ramify extensively in the dorsal direction and sometimes penetrate lamina II (Ramón Y Cajal, 1909; Szentágothai, 1964; Schiebel and Schiebel, 1968). There is usually also a single ventral dendrite and a few short longitudinal dendrites (Schiebel and Schiebel, 1968). The dendritic fields of these cells

are therefore cone shaped, with the apex of the cone directed ventrally. Many of these large cells belong to the SCT (Brown et al., 1977b) and PSDC pathway (Brown and Fyffe, 1981).

Lamina V.

In lamina V the cells become even more varied in size (Rexed, 1952). The dendrites of lamina V cells radiate along dorsoventral and mediolateral planes with little or no extentions rostrocaudally (Schiebel and Schiebel, 1968; Brown, 1981). Thus, the dendritic shapes of these cells were described as flattened disks. The dendrites may penetrate as far dorsally as lamina III and as far ventrally as VII. This contrasts with the orientation of most of the neurons in lamina IV.

Lamina VI.

Lamina VI consists of a medial zone with small, packed cells and a lateral zone with slightly larger cells (Rexed, 1952). Schiebel and Schiebel (1968) observed that the dendritic arbors of these cells are arranged much like those in lamina V.

(ii). CLASSIFICATION OF DORSAL HORN NEURONS ACCORDING TO THE DESTINATION OF THEIR AXONS AND THEIR RESPONSE TO NATURAL STIMULI.

a. **Propiospinal neurons.**

It is assumed that recordings from neurons not

specifically identified as ascending tract cells are likely to be from propiospinal neurons. The justification for this assumption is that by far the majority of spinal cord neurons are interneurons (Chung et al., 1984). These authors estimated that in the sacral cord of rat, 97% of neurons are propiospinal and only 1% project to the cervical enlargement. The remaining 2% of cells were motoneurons. An extremely large number of studies have been devoted to the analysis of the effects of natural stimuli on the activity of unidentified dorsal horn neurons, and a number of different types of cell have been identified. These studies have been performed in both spinalized (Cervero et al., 1976; Christensen and Perl, 1970; Hillmann and Wall, 1969; Menétrey et al., 1977; Rèthelyi et al., 1989; Wall, 1967; Woolf and Fitzgerald, 1983) and intact (Bennett et al., 1980; Cervero et al., 1976; Hillmann and Wall, 1969; Kumazura and Perl, 1978; Price et al., 1979; Steedman et al., 1985; Tapper et al., 1973; Wagman and Price, 1969; Wall, 1967) cat (Bennett et al., 1980; Cervero et al., 1976; Christensen and Perl, 1970; Hillmann and Wall, 1969; Rèthelyi et al., 1989; Steedman et al., 1985; Tapper et al., 1973; Wall, 1967; Woolf and Fitzgerald, 1983) rat (Menétrey et al., 1977) and monkey (Kumazura and Perl, 1978; Price et al., 1979; Wagman and Price, 1969).

Many investigators have reported that neurons can be found in laminae III and IV (and also deeper layers) that are activated just by weak mechanical stimuli applied to

the skin (Kolmodin and Skoglund, 1960; Menétrey et al., 1977; Tapper et al., 1973; Wagman and Price, 1969). These non-nociceptive neurons have also been reported in lamina II (Bennett et al., 1980; Kumazawa and Perl, 1978).

Cells which respond to nociceptive stimuli can be divided into two groups. The first type of nociceptive neuron respond to gentle cutaneous stimuli and increase their discharge when the stimulus becomes noxious, and they have been termed nociceptive non-specific neurons (Besson and Chaouch, 1987). Alternative terms for these cells include multireceptive or wide dynamic range neurons. These neurons occur mainly in laminae IV-VI, and predominantly in lamina V (Hillmann and Wall, 1969; Menétrey et al., 1977; Wagman and Price, 1969; Wall, 1967), although they have also been reported in the superficial laminae (Price et al., 1979; Rèthelyi et al., 1989; Woolf and Fitzgerald, 1983). The second type of propiospinal neuron responding to noxious stimuli are nociceptive specific, i.e. they respond exclusively to high threshold stimuli. These cells are found predominantly in lamina I (Cervero et al., 1976; Christensen and Perl, 1970; Kumazawa and Perl, 1978; Menétrey et al., 1977), although they have been described in lamina II (Kumazawa and Perl, 1978; Price et al., 1979; Rèthelyi et al., 1989; Steedman et al., 1985) and deeper regions (Menétrey et al., 1977). Christensen and Perl (1970) have differentiated two groups of nociceptive specific cells; one type responds only to nociceptive mechanical stimulation (unimodal), whereas the other

group responds to intense thermal and mechanical stimuli.

A number of authors have examined the morphology of physiologically identified neurons within the superficial dorsal horn by combining electrophysiological recording with intracellular staining. Within lamina I of the cat, both nociceptive specific (n=4) (Molony et al., 1981) and nociceptive non-specific (n=2) (Rèthelyi et al., 1989) cells have been stained, and all six units were found to be marginal cells of Waldeyer. Within lamina II of cat, rat and monkey, both limitrophes (stalked cells) and central (islet) cells have been labelled (Bennett et al., 1980; Gobel et al., 1980; Light and Kavookjian, 1988; Molony et al., 1981; Rèthelyi et al., 1989; Woolf and Fitzgerald, 1983), and within each group, non-nociceptive, multireceptive and nociceptive-specific units have been reported, although multireceptive units, particularly those of limitrophes, were rare. One of the most significant findings to emerge from these studies was the correlation between the location and response of central cells. Central cells with dendrites mainly in laminae I and IIo were nociceptive specific or, occasionally, multireceptive, whereas central cells with dendrites in laminae IIi and III were low threshold mechanoreceptors (Bennett et al., 1980; Rèthelyi et al., 1989). This suggests that there may be different functional roles for the outer and inner portion of lamina II.

b. **Projection neurons to the brain.**

There are several groups of dorsal horn neurons whose axons project to identified regions of the brain. The following section describes the locations and physiological properties of several of these systems. In the course of the present study two of these pathways were examined, namely the postsynaptic dorsal column pathway and the spinocervicothalamic tract. These systems will be described in detail, whereas the other major ascending tracts will be dealt with more concisely.

1. POSTSYNAPTIC DORSAL COLUMN (PSDC) SYSTEM.

a. Historical background.

The dorsal columns of the spinal cord have long been known to contain a group of axons which originate from the dorsal root ganglia and ascend directly to the dorsal column nuclei (Walker and Weaver, 1942). In addition, the presence of non-primary afferent (postsynaptic) axons has been known for about a century (Reviewed by Nathan and Smith, 1959), but they were regarded as belonging to propiospinal neurons. However, in 1968, Uddenberg described for the first time the presence of postsynaptic axons in the dorsal columns which project to the brainstem. These cells have since become known as postsynaptic dorsal column, or PSDC, neurons. Later electrophysiological recordings (Angaut-Petit, 1975a) have shown that PSDC fibres represent 9.3% of the total population of myelinated fibres in the fasciculus

gracilis at T12 in the cat. Furthermore, they form 87% of the ascending second-order axons in that segment.

b. Termination of PSDC neurons in the brainstem.

The termination of the PSDC system has been investigated by Rustioni (1973, 1974) using cats that first had chronic dorsal rhizotomies over the length of either the lumbosacral or the cervical enlargement. Subsequent section of the dorsal columns then produced degeneration of the second-order axons exclusively, and their sites of termination could be assessed. The PSDC pathway was found to innervate the dorsal column nuclei, with a small projection to the external cuneate nucleus and nucleus z. This pathway therefore constitutes a spinomedullary projection. Rustioni observed that the pathway was somatotopically organized, with cells in the lumbosacral enlargement projecting mainly to the nucleus gracilis, and cells in the cervical cord projecting mainly to the nucleus cuneatus. This somatotopy has also been reported in the rat (Giesler et al., 1984). Rustioni further noted that the primary afferent and the postsynaptic inputs to the dorsal column nuclei were distributed differently. The terminal arbors of PSDC neurons are confined to the rostral areas of the nucleus, away from the cluster region where the primary afferents terminate. In rats, however, the second-order projection is more much more widespread and PSDC terminals are intermingled with primary afferent axons (Cliffer and

Giesler, 1989).

c. Origin of the PSDC pathway in the dorsal horn.

The numbers, laminar position and morphology of PSDC cells have been studied using the retrograde horseradish peroxidase (HRP) transport technique and intracellular HRP staining.

Laminar location of PSDC neurons.

Cells retrogradely labelled following the application of HRP into the dorsal column nuclei are found almost exclusively in the upper cervical cord and the cervical and lumbosacral enlargements. Very few cells are located in the thoracic cord below T1 (Enevoldson and Gordon, 1989a; Giesler et al., 1984; Rustioni, 1977; Rustioni and Kaufman, 1977).

In the dorsal horn of cat (Enevoldson and Gordon, 1989a; Rustioni and Kaufman, 1977) and rhesus monkey (Rustioni, 1977), PSDC cells are found largely in lamina IV and medial laminae III and V throughout the the cord, with additional cells in medial lamina VI within the high cervical segments. Bennett et al. (1983) also found many PSDC neurons in laminae III-V of the cat lumbosacral enlargement following the application of HRP to the severed ends of axons in the dorsal columns. However, these authors also observed a second group of labelled cells in laminae VII and VIII that comprised 25% of their total. This latter group of labelled cells probably represents a long propiospinal projection. In the rat,

many more PSDC cells are located dorsally in lamina III, particularly in the cervical segments (Giesler et al., 1984).

PSDC neurons have also been stained by intracellular injection of HRP (Bennett et al., 1984; Brown and Fyffe, 1981). The somata of these cells were located in laminae III-V.

Numbers of PSDC Neurons.

Following implantation of HRP into the dorsal column nuclei of cat, Enevoldson and Gordon (1989a) estimated figures of 800-1,000 and 1,700-2,000 cells on each side of the lumbosacral and cervical enlargements respectively. These figures are slightly larger than those given by Giesler et al. (1984) for the rat. These authors estimated 500-700 cells in L4-L6 and 750-1,000 in C6-C8 on one side of the cord.

Bennett et al. (1983) compared the size of the lumbosacral PSDC system in cats and monkeys by placing HRP onto their severed axons in the thoracic dorsal columns. The size in each species was found to be similar, but the cell counts were lower than the estimate of Enevoldson and Gordon (1989a) for the cat.

To compare the relative sizes of the projections to the dorsal column nuclei from PSDC cells and primary afferents in the rat, Giesler et al. (1984) counted labelled cells in the cervical and lumbosacral enlargements and their corresponding dorsal root ganglia

following the injection of HRP into the dorsal column nuclei. PSDC neurons constituted 38% of the neurons that projected to the cuneate nucleus and 29% of the cells that projected to the gracile nucleus.

Morphology of PSDC neurons.

Several authors have attempted to classify PSDC neurons according to the morphology of their dendritic trees. The intracellular staining experiments of Brown and Fyffe (1981) and the retrograde labelling experiments of Enevoldson and Gordon (1989a) have identified 3 morphological subtypes. Brown and Fyffe classified their neurons into types 1-3, which correspond to types A-C in the Enevoldson and Gordon study.

Type 3 cells are the most numerous and are located in lamina IV. They have many dorsally directed dendrites, which sometimes reach into lamina II. They are restricted mediolaterally, but have a relatively prominant rostrocaudal development compared with the other cell types.

The next most numerous type (type 2) have their somata in lamina V or deep IV. These neurons have long straight dendrites that radiate from the cell body in the dorsoventral and mediolateral planes. There is little development rostrocaudally.

Type 1 cells have somata in lamina III or at the III/IV border and have dendritic trees that are particularly well developed dorsally, but which are restricted in both mediolateral and rostrocaudal

directions. These cells send their dendrites into lamina II and sometimes into lamina I.

The above patterns were not confirmed by Bennett et al. (1984) in an intracellular staining study. Bennett's group described a much more extensive rostrocaudal span of the dendritic arbors and proposed that fewer cells send their dendrites into the superficial dorsal horn.

d. Pathway in dorsolateral funiculus to dorsal column nuclei.

Some neurons that ascend to the dorsal column nuclei (DCN) travel in the dorsolateral funiculus (DLF). The lesioning studies of Giesler et al. (1984), in the rat, suggest that about 8% of spinal neurons reaching the DCN do so by means of this route. In agreement, Enevoldson and Gordon (1989b) labelled only a small number of these cells when they implanted HRP-containing gel in the dorsal column nuclei of cats after sectioning the dorsal columns. However, these authors suggested that the poor labelling could also be explained by the DCN collateral being only one of many issued by these cells. Thus, any HRP taken up from the DCN would be diluted in the axoplasmic flow from the other collaterals. These authors noted that labelled axons were quite numerous and well filled until they reached C2, the level of the lateral cervical nucleus (LCN), where the visible label disappeared. Furthermore, a few labelled axons were seen running through the LCN. It was suggested that some of

these spino-DLF-DCN cells might also project to the LCN, and are therefore spinocervical tract (SCT) cells. Jiao et al. (1984) have reported that injection of different fluorescent dyes into the LCN and DCN leads to double staining of some dorsal horn neurons. Furthermore, Craig and Tapper (1978) have found that stimulation of the DCN can excite up to 23% of LCN neurons, and in some cases it was possible to demonstrate, by collision, that this occured via collaterals of SCT axons. Thus, at least some SCT fibres maintain collaterals within the DCN.

e. Primary afferent input to PSDC neurons.

Uddenberg (1968) gave the first indication of the receptors which activate PSDC cells. Uddenberg's sample of units, with receptive fields on cat forelimb, all responded to hair movement and gentle pressure, and gave an increased discharge to pinching the skin. These neurons were therefore multireceptive, receiving convergent input from a wide range of skin receptors.

Feline PSDC neurons with hindlimb receptive fields do not form a homogenous group with regard to response properties. Angaut-Petit (1975b) and Brown et al. (1983) were able to divide their samples of units into three groups. Most neurons (62-77%) responded to a wide range of cutaneous stimuli from hair movement to noxious mechanical and thermal stimuli. Between 16 and 36% were activated solely by light mechanical stimuli and a few units (2-7%) were excited only by noxious pinch. In rat (Giesler and Cliffer, 1985), most lumbar PSDC neurons

(64%) were found to respond only to innocuous mechanical stimuli, and the remaining 36% were multireceptive. These findings show that very few PSDC neurons are excited solely by noxious stimuli and it has been suggested (Giesler and Cliffer, 1985) that this pathway is not importantly involved in nociception.

In addition to cutaneous input, Uddenberg (1968) observed excitation upon bending of the joints and electrical stimulation of high threshold axons in muscle nerves. Jankowska et al. (1979), in an intracellular study, have also recorded input from low threshold (group I) muscle afferents, and estimate that 50% are monosynaptically coupled to PSDC cells. These authors also estimated that 75% of the low threshold cutaneous afferents that excite PSDC cells are monosynaptic coupled. In accord with these findings, Maxwell et al. (1985) have demonstrated morphological monosynaptic contacts between some intra-axonally stained primary afferents and retrogradely labelled PSDC neurons.

f. Descending inputs.

Results from electrophysiological studies in cats have shown that PSDC neurons are regulated by descending systems from the brain. The interuption of descending activity produced by cooling the cord (Noble and Riddell, 1989) or cord transection at C1 (Angaut-Petit, 1975b), markedly increased the sensitivity of PSDC cells to noxious cutaneous stimulation and also greatly amplified

their background discharge. Excitation generated by innocuous cutaneous stimuli was little affected. In addition, cold block expanded the size of the receptive fields of these cells (Noble and Riddell, 1989).

It is possible that there is an inhibitory projection from serotonergic neurons in the brainstem onto PSDC neurons, since these cells have been shown to receive synaptic contacts from axon terminals immunostained for serotonin (Nishikawa et al., 1983; Wu and Wessendorf, 1992).

g. Other inputs.

There is a segmental input to PSDC cells, since inhibition evoked in PSDC cells by cutaneous stimulation is unaffected by cold block (Noble and Riddell, 1989). Maxwell (1988) has observed GABA-containing boutons synapsing upon PSDC neurons, which could mediate at least some of this postsynaptic inhibition.

PSDC cells have also been shown to receive input from the spinocervical tract (SCT). Jankowska et al. (1979) have observed that stimulation of the SCT can evoke excitatory potentials in PSDC neurons. In addition, Maxwell and Koerber (1986) have demonstrated synaptic contacts between SCT axon collaterals and PSDC neurons.

h. Receptive field properties.

In contrast to the receptive fields of most other dorsal horn neurons, which are usually simple, those of PSDC cells are often very complex. For instance, in the

studies of Brown's group (Brown and Fyffe, 1981; Brown et al. 1983), 23 % of neurons had spatially discontinuous receptive fields, which were composed of two or more regions separated by skin from which no excitation can be evoked. In addition, up to one-third of cells had labile receptive fields that expanded in size during the recording period. Occasionally, these new components shrank or disappeared after a period of rest.

i. Correlation between structure and function.

Brown and Fyffe (1981) found several correlations between structure and function for PSDC neurons. Where a cell responded to light mechanical stimulation of the glabrous skin, indicating input from rapidly adapting mechanoreceptors or Pacinian corpuscles, then the dendritic tree was located in the medial part of the dorsal horn, where the primary afferents from these afferents are distributed. Cells with transversely oriented dendritic trees showed some correlation between the transverse spread of the arbor and the size of the receptive field. Neurons with no input from glabrous skin did not have dendrites in laminae V or VI.

2. SPINOCERVICOTHALAMIC PATHWAY.

The spinocervicothalamic pathway was first recognised by Morin (1955) and consists of the spinocervical tract (SCT), which ascends in the dorsolateral funiculus (DLF)

and terminates in the ipsilateral lateral cervical nucleus (LCN), and the neurons of the LCN which project to the contralateral thalamus.

THE SPINOCERVICAL TRACT (SCT).

a. Location of cells of origin.

Brown et al. (1980a) have mapped the location of SCT neurons in the cat lumbosacral enlargement (L4-S2) after flooding the LCN with HRP. The distribution of the retrogradely labelled cells was then compared with the positions of electrophysiologically identified SCT neurons on the other side of the cord. Results from both of these experiments were in excellent agreement, with the exception that the retrograde method labelled a significant population of cells in lamina I. These marginal cells were suggested, at least in the main part, not to belong to the SCT, and when they were ignored, then the electrophysiological and anatomical methods agreed very closely. The vast majority of SCT cells were found on the ipsilateral side (25% in lamina III, 60% in lamina IV, 10% in lamina V and 5% in laminae deeper or more superficial), but a few cells were observed contralaterally.

Enevoldson and Gordon (1989b) obtained similar results after retrogradely labelling cells in cats with HRP from implants in the DLF at C4-C5. These authors also examined labelled neurons in the cervical enlargement and thoracic cord, and found that like those in the

lumbosacral segments, they lay predominantly in laminae III and IV. Other anatomical (Craig, 1978) and electrophysiological (Cervero et al., 1977) studies in cats agree with these findings.

Similarly, in rats, injections of HRP into the LCN labelled many cells in the "nucleus proprius" (laminae III-IV), as well as a few laminae I and II neurons (Giesler et al., 1978). Bryan et al. (1974), using reconstructions of recording sites, have estimated that 80% of primate SCT cells are located in laminae IV and V.

Intracellular staining of identified SCT neurons in cats (Brown et al., 1977b) has confirmed that virtually all of these cells are located in laminae III-V, although an occasional SCT neuron has been stained in lamina I (Maxwell and Koerber, 1987).

b. Numbers of SCT cells.

After HRP injections that flood the LCN, Brown et al. (1980a) concluded that the cat lumbosacral enlargement (L4-S2) contains 550-800 SCT neurons on each side.

When feline SCT axons are sampled from the cervical cord (Heath, 1978) about 50% have forelimb fields, 34% hindlimb fields and 16% fields on the trunk and tail. Using a count of 750 cells in the lumbosacral enlargement, which represents the hindlimb, Brown et al. (1980a) used Heath's figures to estimate that about 1,100 cells will represent the forelimb and 350 will represent the trunk and tail.

Enevoldson and Gordon (1989b) have labelled SCT cells following HRP implants in the DLF at C4 or C5. These authors found 700, 450 and 1,100 neurons in the lumbosacral enlargement (L4-S2), upper lumbar and thoracic cord (L3-T2) and cervical enlargement (T1-C5) respectively: numbers which agree well with the results and calculations of Brown et al. (1980a) above.

c. Morphology of SCT cells.

Organization of dendritic trees.

The published data suggests that although there is variation in the pattern of dendritic arborizations, all SCT cells have certain features in common (Brown et al., 1977b; Enevoldson and Gordon, 1989b). SCT cells in the lower lumbar and upper sacral segments (L6-S2) have been particularly well studied.

In general, the dendritic arbors of SCT cells extend for much greater distances rostrocaudally than mediolaterally, although the maximum rostrocaudal extent depends upon the position of the cell body in the dorsal horn. Cells lying laterally have the greatest rostrocaudal spread (1.4-2.2mm), while cells in the medial third have the smallest (0.5-1.0mm). In the cervical enlargement, SCT cells have a similar morphology, with a corresponding variation from lateral to medial across the dorsal horn (Enevoldson and Gordon, 1989b). SCT cells in the upper lumbar and thoracic segments have elongated perikarya, with primary dendrites originating from their

ends and extending for even greater distances rostrocaudally than their cervical or lower lumbar counterparts (Enevoldson and Gordon, 1989b).

The dendritic trees of SCT cells are usually much better developed dorsally than ventrally (Brown et al., 1977b; Craig, 1978; Enevoldson and Gordon, 1989b), and they thus resemble the classical lamina IV cells of Schiebel and Schiebel (1968). A striking feature of the majority of these dorsally projecting dendrites was that they ascended to the lamina II/III border and then ran rostrocaudally for several hundred μm. Only rarely did SCT dendrites penetrate into lamina II. An important consequence is that these cells would not get direct input from most fine fibres. Many SCT cells have dendritic spines (Brown et al., 1977b).

SCT axons.

Although the initial axonal course is tortuous, all SCT axons eventually reach the ipsilateral DLF and, in cats, ascend in its most superficial and medial part (Brown et al., 1977b; Craig et al., 1987; Enevoldson and Gordon, 1989b). In rats, SCT fibres have been shown to ascend in the lateral part of the DLF (Giesler et al., 1988). Most SCT axons give off local collaterals which arborize in laminae III-VI of the same segment.

d. <u>SCT projections to the LCN.</u>

Degeneration studies (Ha and Liu, 1966; Giesler et

al., 1988) and anterograde tracing studies (Craig et al., 1987; Svensson et al., 1985a) have shown that SCT axons terminate in the LCN. The input to the LCN is chiefly ipsilateral, although in most experiments a small contralateral projection was also found. Some of the fibres entering the LCN were shown to be collaterals of fibres that continue to ascend in the DLF to the brainstem (Ha and Liu, 1966; see also Enevoldson and Gordon, 1989b). At least some of these synapse in the DCN (see PSDC pathway section d).

It has been shown repeatedly, using anterograde labelling, that the SCT projection in cats is somatotopically organized (Broman et al., 1990; Craig et al., 1987; Svensson et al., 1985a). In cats receiving cervical injections of HRP or lectin-conjugated HRP, labelling was concentrated in the ventromedial half of the LCN. In contrast, after lumbar injections the label was detected mainly in the dorsolateral parts of the nucleus. Labelling from thoracic injections occured mainly in the middle of the nucleus. This somatotopy has also been reported in electrophysiological experiments in cats (Craig and Tapper, 1978; Kajander and Giesler, 1987). However, SCT inputs to the medial-most part of the feline LCN is not somatotopically organized, since neurons in this region receive axons from both lumbar and cervical segments (Svensson et al., 1985a; Craig et al., 1987). It was shown recently that lamina I SCT cells project almost exclusively to this region, although its total SCT input is derived from lamina I and laminae III-

V neurons (Craig et al., 1992). Studies in rats have failed to find any evidence of somatotopy in the SCT input to the LCN (Giesler et al., 1979b; 1988), which suggests that there are species differences in the organization of SCT afferents to the LCN.

e.Physiological properties of SCT neurons.

The most important physiological features of SCT neurons are the powerful influences exerted by hair receptors and by descending inhibitory pathways.

Primary afferent input to SCT cells.

Brown and Franz (1969), in a detailed study, have investigated the cutaneous receptors that excite SCT cells in the cat. It was shown that the properties of these neurons depends upon the animal preparation used. In cats having a spinal transection at C1 (thus eliminating tonic descending inhibition), three classes of neurons were recognised according to their responses to mechanical stimuli: (1) Units excited by hair movement alone (30% of the sample). Movement of all types of hair (down, guard and tylotrichs) was effective in activating these cells. (2) Units excited by hair movement and pressure (48% of the sample). Again activity in all three types of hair follicle afferent was an effective excitatory stimulus, as was pressing or squeezing the skin, which was presumably due to nociceptor stimulation. (3) Units excited only by pressure and pinch applied to

the skin and subcutaneous tissues (22% of the sample). It appears, therefore, that in spinalized cats SCT neurons can be classified as low threshold (30%), wide dynamic range (48%) or high threshold units (22%).

Five types of unit were recognised in anaesthetized or decerebrate cats (preparations in which the descending systems are intact): (1). units excited by tylotrichs (16%); (2). units excited by guard hairs and usually, but only weakly, by pressure and pinch (11.5%); (3). units excited by all three types of hair and, often, also by pressure (64.5%); (4). units excited by pressure and pinch (0.5%) and (5). units not affected by peripheral stimulation (7.5%). These reults show that in intact preparations only a tiny minority of SCT cells respond to high threshold stimuli. Thus, although many SCT cells receive connections from nociceptors (22% in spinalized cats), the majority of these inputs are under powerful descending inhibition, and a role for the SCT in nociception is unlikely unless this tonic descending inhibition can be removed.

In a later study, Brown (1971) examined the relationships between SCT cells within these two groups using reversible cold block spinalization. Most units fell into one of four groups. (1) Type I cells (15%); excited only by tylotrichs in the intact (decerebrate) cat, but by all types of hair after block of descending impulses. (2) Type II cells (7%); excited by movement of guard hairs (and usually weakly by pressure) in the decerebrate state, but by all types of hair and pressure

in the spinal state. (3) Type III cells (73%); excited by movement of all types of hairs (and sometimes pressure) in the decerebrate cat, but by all types of hair and always pressure after spinalization. (4) Type IV cells (5%); activated by pressure and pinch or have no receptive field in the decerebrate animal, but are always excited by pressure on release from descending control.

Most SCT units that could be excited by hair movement could also be excited by noxious heat (Brown and Franz, 1969; Cervero et al., 1977), but they were less responsive in decerebrate or anaesthetized cats compared to spinalized animals. Thus, the thermonociceptive input to the SCT is also under a descending inhibitory tone. Kunze et al. (1987) have shown that some feline SCT cells, located in the medial part of the dorsal horn, have receptive fields on the footpads which respond to brush. Furthermore, Harrison and Jankowska (1984) have demonstrated an excitatory input from Pacinian corpuscles.

Brown et al. (1975) have shown in spinalized cats that 30% of SCT cells, with cutaneous receptive fields, receive input from A fibres alone, while 70% are excited by both cutaneous A and C fibres. Units receiving only A fibre input were activated only by hair movement (Type I units), whereas SCT units which responded to A and C fibre stimulation were additionally excited by pressure and pinch (Type II and III units). No type IV units were encountered. By combining intracellular SCT recording

with electrical stimulation of cutaneous nerves, it has been demonstrated that low threshold (Aαß) cutaneous inputs to the SCT from hairs are monosynaptically coupled (Harrison and Jankowska, 1984; Hongo and Koike, 1975), whereas the nociceptor input is probably polysynaptic. In accord, Brown and Noble (1982) have demonstrated morphological monosynaptic contacts between hair follicle afferents and SCT cells that share the same receptive field.

In addition to their cutaneous input, feline SCT neurons have been shown to receive a major excitatory input from muscle nociceptors (Group III and IV afferents), at least in the spinal state (Hamman et al., 1978). This data, obtained by electrically stimulating high threshold axons in muscle nerves, is consistant with results obtained by injecting algesic chemicals (serotonin, bradykinin, KCl) into the gastrocnemius muscle, in order to activate group III and IV muscle afferents (Hong et al., 1979). In these latter experiments, reversible spinalization resulted in a greater number of SCT cells responding to the noxious stimulus (83% versus 39% in the intact cat), and also led to an increase in the magnitude of the response. Group II muscle afferents have been shown to excite a few SCT cells (Hamman et al., 1978; Harrison and Jankowska, 1984), as have high and low threshold joint afferents (Harrison and Jankowska, 1984). Some receptors do not appear to excite SCT cells, at least not in the cat. Cervero and Iggo (1978) found no evidence for an input

from the urinary bladder, and several groups have concluded that group I muscle receptors do not affect SCT cells (Hamman et al., 1978; Harrison and Jankowska, 1984).

All of the information presented so far in this section comes from the large number of studies made of lumbosacral SCT units in cats. Studies of the SCT in other species are sparse. However, Bryan et al. (1974) and Downie et al. (1988) have examined some SCT units in primates. Like those in the cat, most units could be activated by hair movement, and about half could additionally be excited by noxious pinching of the skin.

Receptive field organization of SCT cells.

It has been shown that the cutaneous input to the SCT is somatotopically organized. Cells in the medial dorsal horn have receptive fields on the toes, whereas lateral cells have fields on the foot, leg and thigh (Brown et al., 1980a; Cervero et al., 1977).

Brown et al. (1980a) have proposed that SCT neurons with similar receptive fields are organized into rostrocaudally oriented columns. That is, the receptive fields of neurons within a column overlap. This organization into sagittal columns is a reflection of the input from single hair follicle afferent fibres (Brown et al., 1977c), as each hair follicle afferent forms a sagittal sheet of terminals that project onto several SCT cells. In an intracellular staining study, Brown et al.

(1980b) have found that SCT cells with overlapping receptive fields have interdigitating dendritic trees, whereas adjacent pairs of cells with separate receptive fields have dendritic arbors that do not overlap.

As already mentioned above, Brown and Noble (1982) have demonstrated anatomical contacts between intracellularly labelled hair follicle afferents and SCT cells with overlapping receptive fields. Moreover, it was observed that when the afferent's field was centrally placed in the SCT field, then there were many contacts on the soma and proximal dendrites, but when the afferent's field was located peripherally, then there were fewer contacts which were located on distal dendrites. Functionally, this means that primary afferents with centrally placed fields give rise to larger EPSP's with faster rise times than fibres with peripherally placed fields (Hongo and Koike, 1975).

Descending inputs to SCT cells.

The differences between spinalized and intact cats in the response properties of their SCT neurons (Brown, 1971; Brown and Franz, 1969; Hong et al., 1979) shows that powerful descending systems, active in anaesthetized and decerebrate animals, operate to control the input to the SCT. The descending inputs make SCT cells less sensitive to noxious stimuli, and also make them more restricted in their hair input. For example, in Type I and II units (Brown, 1971) they depress all excitatory inputs except those from a single category of hair

afferent (types T and G respectively). Brown et al. (1973, 1975) have further shown that stimulating the DLF and ventral quadrant at C2-C4 in spinalized cats, (to stimulate the descending pathways), can inhibit input to the SCT. The descending inhibition was particularly effective upon polysynaptic responses evoked by small myelinated and unmyelinated fibres, but had little or no effect on monosynaptic responses evoked from large diameter myelinated afferents.

Although it is clear that transmission through the SCT is inhibited by pathways originating in the brain, the mechanisms underlying this control are largely unknown. However, the selectivity of the actions suggests that the pathways do not act on the SCT cells themselves, but on interneurons in polysynaptic pathways to the SCT or upon primary afferent terminals. A presynaptic action is suggested by the long time course of the descending inhibition (>200ms) (Brown et al., 1973; Brown and Martin, 1973; Brown and Short, 1974).

More recently, the origins of some of these descending pathways have been elucidated. The SCT has been shown to be under inhibitory control from the sensorimotor cortex (Brown and Short, 1974). These authors demonstrated, in chloralose anaesthetized cats, that electrical stimulation of the first and second somatic sensory areas (SI and SII) profoundly inhibits SCT responses to electrical stimulation of cutaneous nerves. This inhibition was almost completely abolished by bilateral

section of the DLF. With microstimulation, it has been shown that these effects can be evoked from cytoarchitectonic areas 3a, 3b and 1 of SI, areas 4γ and 4δ of motor cortex and from areas 5a and 5b of the parietal association cortex (Brown et al., 1977a). Fleetwood-Walker et al. (1988) have observed that stimulation of the A11 dopamine cell group in rats selectively suppressed the nociceptive responses of wide dynamic range SCT cells. This inhibition was blocked by sulpiride, a D-2 dopamine receptor antagonist. Inhibitory effects on the SCT have also been observed following electrical stimulation in the region of the periaquductal grey and the nucleus raphe magnus (Gray and Dostrovsky, 1983; Kajander et al., 1984). These loci inhibited both noxious and non-noxious inputs to SCT cells. There does not appear to be any descending effects on the SCT from the A9 dopamine cell group (Fleetwood-Walker et al., 1988) or the red nucleus (Harrison and Jankowska, 1984).

THE LATERAL CERVICAL NUCLEUS (LCN).

a. Taxonomic distribution and topography.

The LCN is prominant in the upper cervical spinal cord in a variety of mammals, but it is larger in carnivores, such as cats and dogs, (Craig and Burton, 1979) than in primates (Ha, 1971). In these species, the LCN is present throughout the first two cervical segments and consists of a column of grey matter in the dorsolateral funiculus (DLF), just lateral to the dorsal horn.

Rostrally, the LCN extends slightly into the medulla (Craig and Burton, 1979; Flink and Westman, 1986). Proceeding caudally, it elongates mediolaterally to become a prominant comma-shaped nucleus in C1 (Craig and Burton, 1979; Maxwell et al., 1989). The nucleus then gradually diminishes as it nears the C2-C3 border.

A long column of cells in the DLF, that extends the entire length of the spinal cord, has been reported in several species, including rat. It was suggested that this structure performs the functions served by the LCN in other species (Gwyn and Waldron, 1968). However, more recent anatomical and physiological findings indicate that the DLF of the rat contains two distinct nuclei: the LCN and the lateral spinal nucleus. The cells of the LCN lie in the lateral DLF of segment C2 (Giesler and Elde, 1985), receive input from SCT axons (Giesler et al., 1988) and project to the thalamus (Giesler et al., 1979a). The lateral spinal nucleus comprises those cells in the medial DLF of C2 as well as those found throughout the DLF of segments caudally (Giesler and Elde, 1985). The lateral spinal nucleus does not receive SCT axons (Giesler et al., 1988) nor does it project to the thalamus (Giesler et al., 1979a).

The presence of an LCN in humans is still a matter of controversy. Boivie (1983) suggests that its appearance is highly reticulated, resembling a coarse mesh, and suggest that this may help explain why many investigators have failed to find the LCN in man.

b.Morphology of LCN neurons.

Most cell bodies in the cat LCN are fairly large (30-50μm), although some have diameters of 20μm or less (Westman, 1968b). The small cells are found mainly in the medial part of the nucleus, and form about 30% of LCN neurons, whereas the large cells are mainly distributed in the lateral two-thirds of the LCN (Craig and Burton, 1979).

LCN cells have been shown using the Golgi technique to have a characteristic appearance (Ha and Liu, 1966; Westman, 1968a). They have 4-9 main dendrites (mostly 6) which branch repeatedly and are beset with spines. The dendrites seldom extend beyond the borders of the nucleus and are mainly oriented rostrocaudally. Some cells at the border between the LCN and the dorsal horn are atypical. The axons of LCN neurons have been shown to give rise to local collaterals, but with Golgi staining it has not been possible to follow any axon to its projection site. However, well-labelled axons from retrograde tracing experiments (Craig and Burton, 1979) and axons exhibiting anterograde degeneration (Ha, 1971; Boivie, 1980) could often be followed for considerable distances. LCN axons pass through the intermediate zone, cross in the ventral commissure, and ascend in the ventromedial part of the ventral quadrant to join the medial lemniscus in the medulla.

c.Numbers of LCN neurons.

The total number of neurons in the feline LCN has been

estimated in frozen sections to be 4,800-7,450 (Craig and Burton, 1979). More recent calculations in semithin plastic-embedded sections have indicated that the number may be 7,800-9,100 (Flink and Westman, 1986). In rats, the LCN contains 300-500 neurons (Baker and Giesler, 1984).

d. Ultrastructure of LCN neurons.

The ultrastructural appearance of LCN neurons has been described by Westman (1968b). A large part of the somatic and dendritic surfaces were covered with synaptic boutons, most of which were about 1μm diameter. The majority of these contained round synaptic vesicles, but terminals with elongated vesicles were also found. A special type of terminal, an elongated giant bouton with dimensions up to 10μm X 3μm, was found contacting the dendrites of LCN neurons. These terminals were relatively rare though.

After interupting the SCT, by lesioning the DLF, the giant boutons disappeared, as did many smaller terminals (Westman, 1969). The degenerating boutons were found in association with large (30-50μm), but not small (<20μm) LCN neurons. Svensson et al. (1987) have examined SCT terminals in the LCN that were anterogradely labelled with lectin-conjugated HRP. These boutons contained round or oval synaptic vesicles and formed synapses with dendrites (84%) and somata (16%). Broman et al. (1990) showed that these SCT terminals were enriched with

glutamate. A few SCT boutons in the LCN, which contained flattened vesicles (Svensson et al., 1987), were not glutamate-immunoreactive (Broman et al., 1990). Svensson et al. (1987) have estimated that SCT terminals comprise 14% of the bouton volume of the LCN, and that each SCT axon gives rise to an average of 4,400 boutons.

e. Immunocytochemistry of the LCN.

Immunocytochemical investigations have shown the presence of several transmitter or transmitter related substances in the LCN. The presence of glutamate-immunoreactive SCT afferent terminals (Broman et al., 1990) has already been mentioned.

The presence of GABA-containing terminals in the LCN has also been established (Blomqvist et al., 1985; Broman and Westman, 1988; Maxwell et al., 1989), and these structures are scattered throughout the nucleus. Ultrastrutural analysis showed that these GABAergic terminals were densely packed with irregularly shaped vesicles and that they formed symmetrical synapses with dendrites and somata. GABA-positive neurons have also been demonstrated in the LCN (Broman and Westman, 1988). These cells were small and concentrated in the medial part of the nucleus. The authors found 40-50 cells in the LCN on each side, and suggested that they function as LCN interneurons.

Substance P-immunoreactive axons are present in the LCN of cat, rat and primate, but their distribution differs between species. They occur throughout the LCN

of rat and monkey, but are restricted to the ventromedial part of the nucleus in cats (Blomqvist et al., 1985; Broman and Blomqvist, 1989). The ultrastructural features of substance P-positive boutons are similar to those of labelled SCT terminals (Svensson et al., 1987), and Broman and Blomqvist (1989) have suggested that the most likely source of substance P in the LCN is from SCT neurons. However, recent double-labelling studies by Craig et al. (1992) have shown that only a small number of substance P-immunoreactive fibres in the feline LCN originate from the SCT. It now seems likely that they belong to descending systems.

A network of serotonin-containing fibres is present in the LCN of cats and monkeys, but the density of labelled fibres is greater in the monkey (Broman and Blomqvist, 1990). These terminals contain densely packed, round synaptic vesicles and only rarely form synaptic specializations. This innervation probably also originates from supraspinal sites.

To date, there is no evidence for the existence of axo-axonic synapses in the LCN.

f. Projection targets of LCN neurons.

The main projection targets of the LCN are the contralateral thalamus and mesencephalon. There is also a small ipsilateral projection to the spinal cord.

Cervicothalamic tract (CTT).

Anterograde degeneration studies have shown that the main thalamic nuclei which receive CTT terminals are the lateral & medial parts of the nucleus ventralis posterolateralis (VPLl and VPLm) and the medial part of the posterior nucleus complex (POm) (Boivie, 1970; 1980; Giesler et al., 1988). The CTT projection in monkey (Boivie, 1980) and rat (Giesler et al., 1988) are considerably smaller than the equivalent projection in cat (Boivie, 1970).

The distribution of thalamic projection cells in the LCN has been studied by retrograde tracing (Craig and Burton, 1979; Flink and Westman, 1986; Flink and Svensson, 1986; Berkley et al., 1980). CTT neurons were found throughout the LCN, with a slight concentration in the lateral two-thirds. It was observed that most (82-97%) LCN neurons projected to the thalamus, which accords with results from antidromic activation studies (Brown et al., 1993; Craig and Tapper, 1978; Giesler et al., 1979b). CTT neurons were usually large (25-45μm), although some small (<20μm), medially located, LCN cells were also labelled following thalamic injections of HRP (Craig and Burton, 1979; Flink and Westman, 1986). LCN cells which remained unlabelled after large thalamic injections (Craig and Burton, 1979) were predominantly small cells located in the medial part of the nucleus. These cells comprised about 5% of LCN neurons, and the authors suggested that they may be local circuit neurons.

However, subsequent to the publication of this study LCN projections to the mesencephalon, spinal cord and inferior olive were reported, which reduces the likelihood of these cells being interneurons. Flink and Westman (1986) made HRP injections into all of the known projection sites of LCN neurons, and found that about 50 cells were left unlabelled in the medial LCN. These cells were suggested to represent true LCN interneurons. The number and location of these cells is identical to that of LCN neurons stained for GABA (Broman and Westman, 1988), and it is possible that the two cell groups are equivalent.

Craig and Burton (1979) have shown, by injecting small amounts of HRP into different parts of the VPL of cats, that the CTT neurons are somatotopically organized. Dorsolateral LCN cells (which receive input from lumbosacral SCT cells) project to the VPLl and ventromedial LCN cells (which receive input from the cervical SCT) project to the VPLm. In contrast, the CTT in rats is not somatotopically organized (Giesler et al., 1979b).

Cervicomesencephalic tract (CMT).

The CMT terminates mainly in the intercollicular nucleus and the posterolateral part of the superior colliculus (Flink et al., 1983; Giesler et al., 1988). Retrograde labelling studies have suggested that between 28% and 55% of LCN neurons project to the mesencephalon (Berkley et al., 1980; Flink and Westman, 1986),

depending upon the tracer substance used. Higher estimates were achieved with cholera toxin subunit b and fluorescent substances than with HRP.

Several studies have compared retrograde labelling from the thalamus with that produced by simultaneous thalamic and mesencephalic injections. On the basis these results, some authors have suggested that practically all CMT neurons also project to the thalamus (Flink and Svensson, 1986; Flink and Westman, 1986). However, others have reported that a significant population of LCN neurons (12-18%) project to the mesencephalon alone (Berkley et al., 1980; see also Brown et al., 1993).

Cervicospinal projection.

A small descending projection to the ipsilateral spinal cord has been demonstrated by retrograde labelling (Broman et al., 1990; Craig et al., 1977; Flink and Svensson, 1986; Svensson et al., 1985b). There are about 500 descending LCN cells on each side of the cord, with twice as many cells projecting to the cervical segments than to the lumbar region (Svensson et al., 1985b). These cells are quite small (about 25μm) and their axons descend in the dorsomedial DLF (Svensson et al., 1985b). Flink and Svensson (1986) have shown that these descending LCN neurons do not have any ascending projections to the brain.

Cervico-olivary projection.

A scattered cell population in the LCN that projects to the inferior olive has also been reported (Molinari, 1984).

g. Functional studies of LCN neurons.

Responses to electrical stimulation.

Fedina et al. (1968) observed that transmission through the spinocervicothalamic pathway, evoked by DLF stimulation at T12 and recorded from the medial lemniscus, could be inhibited by up to 50% by electrically stimulating the skin or cutaneous nerves. Lesion studies suggested that the inhibitory fibres travel mainly in the contralateral ventral quadrant, and intracellular recordings from LCN neurons showed that postsynaptic, hyperpolarizing IPSP's act on the LCN cells.

Inhibition of LCN neurons has also been recorded after stimulating the medial lemniscus (Gordon and Jukes, 1963). The authors suggested that this might have been due to antidromic activation of the recurrent collaterals of LCN projection neurons.

Primary afferent input to the LCN.

Experiments in barbiturate-anaesthetized cats (Craig and Tapper, 1978; Gordon and Jukes, 1963; Metherate et al., 1986) have shown that most LCN neurons (83-92%) are

excited by hair receptors. The drive from hair receptors was very strong and evoked vigorous responses, just as in SCT cells. About half of these units were also excited by other peripheral receptors (e.g. pressure, field and deep tissue) (Craig and Tapper, 1978). In these experiments, very few LCN units were observed that responded to noxious stimuli (Craig and Tapper, 1978), which was surprising since SCT cells excited by noxious pressure make up about 65% of the total (Brown, 1971; Brown and Franz, 1969). Kajander and Giesler (1987) have shown that this lack of response to noxious stimuli was probably due to the use of barbiturate anaesthesia. In chloralose-anaesthetized cats (Brown et al., 1989) found that 29% of LCN cells were excited by hair movement alone, 65% were additionally excited by noxious pinch, 1% were nociceptive specific and 5% had no cutaneous receptive field. These relative proportions were remarkably similar to those found in the SCT (Brown, 1971; Brown and Franz, 1969).

The response properties of LCN neurons in decerebrated, spinalized cats was investigated by Kajander and Giesler (1987): 41% of cells were low threshold units; 49% were wide dynamic range and 9% were high threshold units. Thus, as with SCT cells, spinalization renders more cells in the LCN nociceptive specific. Similar results to complete spinalization were produced by sectioning just the DL, and thus the descending inhibitory pathways seem to originate in the brainstem and project to the spinal cord via the DLF.

Other peripheral receptors seem to be only weakly represented in the cat LCN. As with the SCT, there was only minor input from slowly adapting mechanoreceptors (SA I and II) and Pacinian corpuscles (Craig and Tapper, 1978; Metherate et al., 1986). The stimulation of receptors below the skin (which may have been muscle afferents) excited only 5% of LCN units (Craig and Tapper, 1978). Furthermore, less than 3% of cells received a crudely defined input from the viscera, and only 1/556 cells responded to joint movement (Craig and Tapper, 1978).

The primary afferent input to the rat LCN has been reported in a study by Giesler et al. (1979b), in animals anaesthetized with urethane. All of the units received excitatory input from hairs and responses were observed to noxious mechanical stimuli in 27% of the cells (most of which were also excited by noxious heat). Several units were activated by intraperitoneal injections of hypertonic saline, suggesting an input from the viscera. In contrast to experiments in the cat (Kajander and Giesler, 1987), spinalization produced no changes in the response properties of rat LCN neurons.

Downie et al. (1988) have recorded from LCN units in primates that were anaesthetized initially with chloralose, but then maintained with barbiturate. Neurons were assigned to the following groups according to their response to mechanical stimuli: low threshold (45%); wide dynamic range (48%); high threshold (7%).

Thus, in primates, unlike cat and rat, many LCN units responded specifically to noxious pinch in the anaesthetized state. About 60% of monkey LCN units also responded to noxious heat (Downie et al., 1988).

Receptive field organization of LCN neurons.

The majority of LCN neurons have receptive fields that are similar in size and shape to those of SCT neurons (Brown et al., 1989; Craig and Tapper, 1978). However, about 10-14% have fields that are obviously larger than those encountered with SCT cells or differ in their shape (Brown et al., 1989; Craig and Tapper, 1978; Downie et al., 1988; Giesler et al., 1979b; Kajander and Giesler, 1987; Metherate et al., 1986). For instance, about 5% of the units studied by Craig and Tapper (1978) had spatially discontinuous receptive fields; two-thirds of which received separate inputs from the fore- and hind-limbs ("wide-field" units). Furthermore, Brown et al. (1989) found a number of units whose fields completely surrounded part of a limb ("stocking-like" fields). These groups of LCN neurons presumably receive convergent input from a number of SCT cells. Quantitative studies have indicated that there are 3-4 fold more neurons in the LCN (Flink and Westman, 1986) than there are SCT cells projecting to it (Brown et al., 1980a; Enevoldson and Gordon, 1989b). Hence, in addition to convergence, there is ample opportunity for divergance of the SCT input too.

Inhibitory receptive fields have been observed for

some LCN neurons. In particular, two types of inhibitory process depress excitation in the LCN. The first type of inhibition ("Out of field" inhibition) can be elicited by stimulating areas of skin outside of the excitatory receptive field (Brown et al., 1989; Craig and Tapper, 1978; Gordon and Jukes, 1963; Kajander and Giesler, 1987). The second type of inhibition comes from within the excitatory receptive field itself ("In field" inhibition), and has been studied in detail by Brown et al. (1989). These authors observed that when two air-jet stimuli were applied 200ms apart, to two points close together in the receptive field, then the response to the second stimulus was markedly inhibited relative to the first. Both "In field" and "Out of field" inhibition have also been demonstrated in the SCT (Noble and Short, 1989), and Brown et al. (1989) concluded that the LCN was simply relaying this pattern of inhibition.

Descending inputs to the LCN.

Little is known about the descending control of LCN neurons. Activity in various brainstem nuclei has been shown to inhibit the response of LCN neurons to cutaneous stimulation (Dostrovsky, 1984). However, since it only weakly affected excitation produced by stimulating the DLF, it was concluded that this inhibition acts on inputs to the SCT. Similarly, Kajander and Giesler (1987) have shown that spinalization renders feline LCN neurons more sensitive to noxious stimuli, but this response may also

be effected at the level of the SCT.

However, Craig (1978) has demonstrated the existence of descending connections from the dorsal column nuclei and several other medullary nuclei to the LCN, but nothing is known about their physiology. Furthermore, Peto (1980) found that stimulating area 3a of the cortex inhibited the excitation generated in LCN neurons by DLF stimulation. Area 3a forms part of the cortical representation of the face and Peto suggested that the inhibitory pathway might be active in situations requiring face-limb co-ordination, such as grooming. A few LCN cells also received monosynaptic excitatory input from the cortex (Peto, 1980).

3. SPINOTHALAMIC TRACT (STT).

Retrograde tracing studies in the rat (Giesler et al., 1979a; Kevetter and Willis, 1983), cat (Carstens and Trevino, 1978) and monkey (Albe-Fessard et al., 1975; Apkarian and Hodge, 1989: Willis et al., 1979) have demonstrated three main zones of origin of STT neurons in the dorsal horn: lamina I, laminae IV-VI and laminae VII-VIII. Despite this wide distribution, however, some differences may be noted with respect to the extent of the STT projections from each of these regions. In the rat, for instance, there are relatively few STT cells in lamina I, while in the cat few STT neurons are found in laminae IV-VI. In all three species the majority of retrogradely labelled cells were found contralateral to

the thalamic injection site. The distribution of STT cells has also been mapped using antidromic activation techniques (Trevino et al., 1972; 1973; Albe-Fessard et al., 1974) and, in general, the locations of identified cells agrees well with the results obtained using retrograde tracers.

The physiological properties of STT neurons have been studied in the monkey by Willis's group (Willis et al., 1974; see also Downie et al., 1988). In the latter of these studies, 318 neurons were tested, and apportioned to the following groups on the basis of their response to cutaneous stimulation: wide dynamic range (59%), nociceptive specific (32%) and low threshold (9%). Thus, the STT differs from the PSDC and spinocervicothalamic pathways in that many of its cells receive input only from nociceptors. The axons of most STT neurons ascend the cord in the ventral quadrant (Jones et al., 1987), and section of this region (ventral cordotomy) can alleviate certain pains, notably those resulting from cancer (Spiller and Martin, 1912). About 11% of primate STT cells receive input from the deep tissues (Willis et al., 1974).

4. Other ascending tracts.

Other direct spinal pathways to the brain include the spinoreticular tract (Kerr, 1975; Kevetter et al., 1982; Kevetter and Willis, 1983), which terminates in the reticular formation, and the spinomesencephalic tract

(Flink et al., 1983; Wiberg et al., 1987), which synapses mainly in the parabrachial nuclei, periaqueductal grey, intercollicular nucleus, superior colliculus, posterior pretectal nucleus and the nucleus of Darkschewitsch.

(d). **Descending input from the brain.**

The dorsal horn, in addition to receiving inputs from primary afferent fibres, also receives inputs from neurons located in the brain. These descending projections include, amongst many others, the cortico-spinal (Brown and Short, 1974; Cheema et al., 1984; Hayes and Rustioni, 1981) and raphe-spinal (Basbaum and Fields, 1979; Martin et al., 1979b) tracts. There is also a major descending input from the catecholaminergic nuclei of the brainstem and midbrain. These latter systems form the basis of this thesis and their properties will now be described in detail.

(2). ORGANIZATION OF DESCENDING CATECHOLAMINE PATHWAYS.

(a). **Biochemistry and Pharmacology.**

Catecholamine (CA) neurotransmitters; dopamine (DA), noradrenaline (NA) and adrenaline (AD) are synthesized from tyrosine within neurons that possess the relevant synthetic enzymes. The biosynthetic pathway is shown in figure 1 for easy reference.

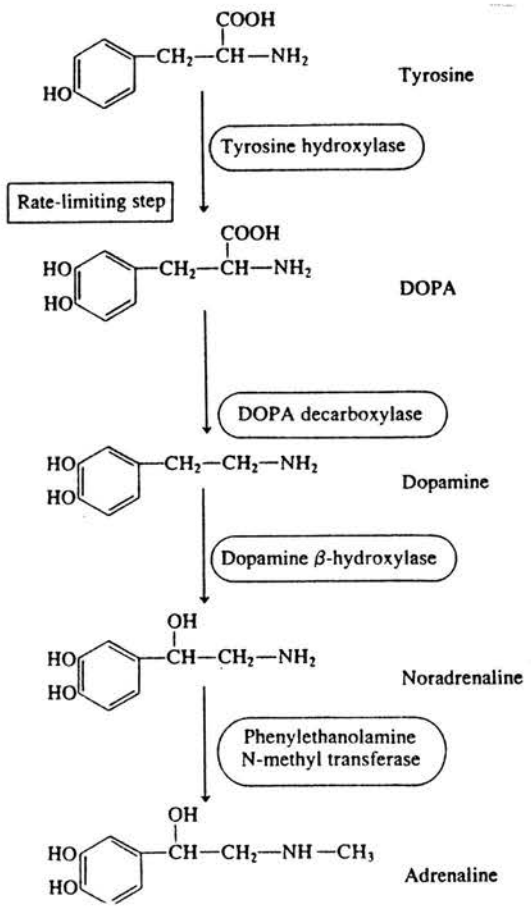


Fig.1 The Biosynthesis of Catecholamines.

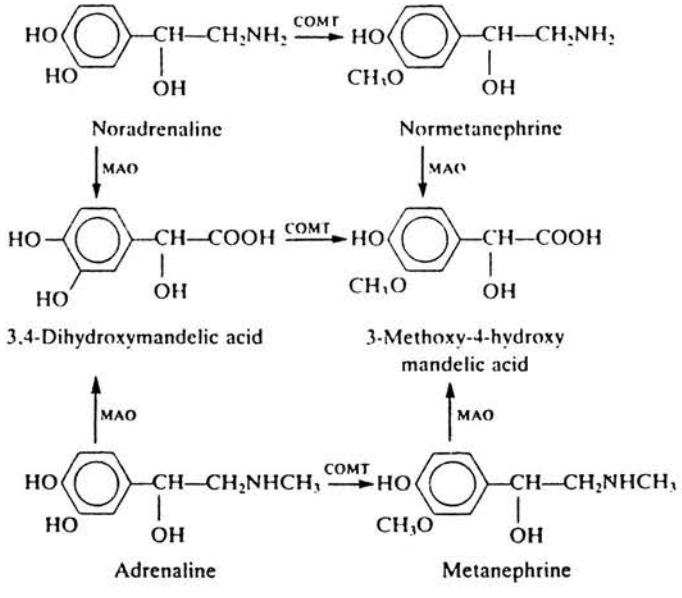


Fig.2 The Metabolism of Catecholamines.

The enzymes in the synthetic pathway are not wholly specific, and drugs such as α-methyldopa are acted on by DOPA-decarboxylase to produce α-methyldopamine, which is hydroxylated to α-methyl-noradrenaline. These derivatives act as "false transmitters", since they replace noradrenaline in storage vesicles, resulting in reduced release of the active transmitter. Similarly, inhibitors of the biosynthetic enzymes are known. For instance, α-methyltyrosine is a potent inhibitor of tyrosine hydroxylase, and dopamine-ß-hydroxylase is inhibited by FLA-63. These agents also lead to a reduction of the CA content of the neurons.

During the last ten years, considerable progress has been made in characterizing the receptors through which CA's act in the central nervous system. Pharmacological studies have indicated that four main classes of receptor (designated α-1, α-2, ß-1 and ß-2) are acted upon by nordrenaline (NA) and adrenaline (NA) (Hamon et al., 1991; Nicoll et al., 1990). These different receptor subtypes are classified according to their interactions with various agonists and antagonists. Table 1 shows the pharmacological basis for the classification of α-adrenoceptor subtypes.

Table 1.

	A. Order of selectivity of agonists.	B. Order of selectivity of antagonists.
α-1	Methoxamine Amidephrine Phenylephrine	Prazosin
α-1=α-2	Adrenaline Noradrenaline Dopamine	Phenoxybenzamine Phentolamine
α-2	Clonidine BHT-920 Dexmedetomidine UK-14304	Yohimbine Rauwolscine Idazoxan

Binding studies with selective radioligands have revealed the presence of approximately equal densities of α-1 and α-2 binding sites in the rat spinal cord (Simmons and Jones, 1988). α-1 receptors are uniformly distributed throughout the grey matter, whereas α-2 receptors are significantly more abundant in the dorsal horn. Pharmacological studies in the spinal cord (see later) indicate that the effects produced by NA (and AD) on sensory transmission, are mediated by adrenoceptors of the α-2 subtype. Recently, α-2 receptors were divided into four further subtypes (α-2A to α-2D), and it was reported that α-2A subtype was the predominant α-2 receptor in human spinal cord (Lawhead et al., 1992). ß-adrenoceptors have also been reported in the spinal cord (Hamon et al., 1991), but the physiological role of this receptor remains uncertain. Two main classes of dopamine (DA) receptor, designated D-1 and D-2, have been identified in the central nervous system, and selective agonists and antagonists that allow their differentiation

are available (Hamon et al., 1991; Nicoll et al., 1990). Apomorphine is a selective agonist at D-2 receptor sites, whereas sulpiride and pimozide are selective antagonists. D-2 receptors are known to mediate the effects of DA in the dorsal horn (see later), and radioligand binding studies have described a dense population of D-2 receptors in this region (Bouthenet et al., 1987).

Following their synaptic release, CA transmitters are rapidly taken back up into the nerve terminal. This re-uptake mechanism is the dominant process in the removal of liberated CA from the synaptic cleft, and its selective inhibition can be achieved using desmethylimipramine and benztropine for noradrenergic and dopaminergic neurons respectively. A very widely used substance in studies of CA pathways is the neurotoxin 6-hydroxydopamine (6OHDA). In low concentrations it is selectively toxic for NA- and DA-containing neurons, as it is transported intracellularly by the re-uptake systems of these cells (Kostrzewa and Jacobowitz, 1974; Sachs and Jonsson, 1975; Thoenen and Tranzer, 1973). The toxic action of this amine effectively results in a pharmacological lesion of the CA-containing pathway.

CA neurotransmitters may be degraded by two major metabolic pathways (see figure 2). These are the methylation of the 3-hydroxyl group of the catechol ring by the enzyme catechol-O-methyl transferase (COMT) and the oxidative removal of their amino group by monoamine oxidase (MAO). 3-O-methylation by COMT leads to the

formation of normetanephrine and metanephrine. This enzyme is located extraneuronally, and is inhibited by the competitive substrates pyrogallol and dopacetamide. MAO is a mitochondrial enzyme, which is inhibited by drugs such as phenelzine and nialamide. Inhibition of this enzyme is frequently used to produce an accumulation of CA at the synapse.

(b). **Histochemistry.**

A major stimulus for studying CA's in the central nervous system came with the development of a histofluorescence technique for localizing these substances in the brain (Carlsson et al., 1962). The method depends upon the formation of fluorescent reaction products (3,4-dihydroisoquinolines) when formaldehyde gas reacts with CA's under certain conditions (Carlsson et al., 1964). This technique is often referred to as the Falck-Hillarp formaldehyde (FA) method, and it is usually specific for NA and DA. Secondary amines such as AD are not converted to fluorescent products to any significant degree under the conditions used. Serotonin, on the other hand, will condense with formaldehyde to produce fluorescent 6-hydroxy-3,4-dihydro-ß-carbolines. However the light emitted by these derivatives differs from that emitted by 3,4-dihydroisoquinolines, so that CA-containing structures appear yellow, while tryptamines are more green. Subsequent work has led to the establishment of glyoxylic acid as a second compound that can be used to demonstrate CA's in the central nervous

system (Lindvall and Björklund, 1974). Glyoxylic acid cyclisation with CA yields a much stronger fluorophore than formaldehyde condensation, and with this compound a more detailed anatomical account of the CA systems has been achieved.

Since the demonstration that CA's are localised in specific neuron systems (Carlsson et al., 1962; 1964; Dahlström and Fuxe, 1964, 1965; Lindvall and Björklund, 1974), these neurotransmitters have received an ever-increasing interest in the analysis of brain and spinal cord function. Histofluorescence techniques, however, have now largely been superceded by more elaborate immunocytochemical methods.

(c). Distribution of brainstem catecholaminergic nuclei.

It is now accepted that the catecholaminergic innervation of the spinal cord is derived from cell bodies located in the brain (see later). A review describing the CA innervation to the spinal cord would be incomplete, therefore, without describing the anatomical organization of these perikarya within the brain.

In their pioneering study, Dahlström and Fuxe (1964) described the distribution of CA-containing perikarya in the rat brain, and classified them into twelve groups, designated A1-A12. These groups occur in the medulla (A1-A4), pons (A5-A7), mesencephalon (A8-A10) and diencephalon (A11-A12). Groups A1-A7 are noradrenergic, while groups A8-A12 are dopaminergic (Lindvall and

Björklund, 1982).

1. Groups A1-A4.

The A1 grouping of cell bodies is located within the ventrolateral aspect of the medulla in the region of the lateral reticular nucleus. The A2 bodies are contained more dorsomedially within the region of the nucleus tractus solitarius. Hökfelt et al. (1974) showed that a proportion of the cells in these groups contain phenylethanolamine-N-methyltransferase (PNMT), suggesting that they are adrenergic. These cells were found in the rostral portion of the A1 and A2 groups, and the designation C1 and C2 respectively, has been introduced for these cells. Because of the paucity of A3 cell bodies, this cell group is no longer recognised as a nuclear entity. The A4 cells constitute a small group caudal to the locus coeruleus (LC), whose cell bodies lie just dorsal and lateral to the fourth ventricle. They appear to be a caudal extention of the LC (A6 cell group).

2. Groups A5-A7.

The A5 group appears as a rostral extention of the A1 cells and is situated just lateral to the superior olive. The LC, originally described as the A6 grouping, is the most discrete and compact mass of NA-containing cell bodies in the brain. The A7 group, like the A5 group, appears to be a rostral continuation of the A1 cells. Anatomically it is subdivided into a number of smaller

nuclei. Some cell bodies are dispersed just beneath the LC, forming the subcoeruleus group. Another cluster of cells lie lateral to the superior cerebellar peduncle in the parabrachial nuclei, while another cluster is found within the Kölliker-Fuse nucleus.

3. Groups A8-A10.

The A8 cell bodies are contained within the nucleus cuneiformis and ventrolateral to the red nucleus. Rostrally, these blend in with the cells of the A9 group, most of which are found within the substantia nigra. The A10 cell bodies are found primarily in the medial region above the interpenduncular nucleus and the ventral tegmental area. There is no sharp demarcation between the A10 and A9 cells.

4. Groups A11 and A12.

The A11 cell bodies are found within the caudal thalamus and in the posterior hypothalamic area medial to the fasciculus retroflexus and the medial lemniscus. The cells of the A12 group are located within the arcuate nucleus.

Later additions to this classification were one cell group in the zona incerta and caudal hypothalamus (designated A13; Fuxe et al., 1969), and another in the periventricular grey of the anterior hypothalamus and the preoptic region (designated A14; Björklund and Nobin,

1973). The A13 group is a rostral extention of the A11 cell group. A further group of cells exhibiting a weak CA fluoroscence has been described in the olfactory bulb (Dahlstöm and Fuxe, 1964; Hökfelt et al., 1975). Halasz et al. (1977) have proposed the designation A15 for this group of cells. These three groups of cells are dopaminergic. A further group of PNMT-positive (adrenergic) perikarya has been reported within the medial longitudinal fasciculus of the medulla (Howe et al., 1980; Sawchenko et al., 1985). These cells have been designated C3.

Similar groups of supraspinal CA-containing perikarya are present in the cat (Lackner, 1980; Poitras and Parent, 1978), rabbit (Blessing et al., 1981) and primate (Hubbard and DiCarlo, 1974; Westlund et al., 1984).

(d). **Catecholaminergic innervation of the spinal cord.**

The presence of CA in the spinal cord was first reported by von Euler (1947) and Vogt (1954). These studies were performed on spinal cord extracts and provided no information about the cellular localization and function of NA in the cord.

In 1962, using the Falck-Hillarp method, Carlsson et al. were the first to report the presence of CA-containing nerve terminals within the spinal cord of the rat, and their findings have been confirmed in many histofluorescence studies since (Dahlström and Fuxe, 1965; Fuxe, 1965; Schroder and Skagerberg, 1985). They have also been extended to other species such as cat

(Lackner, 1980) and primate (Crutcher and Bingham, 1978). CA-containing fibres are rather unevenly distributed throughout the spinal grey matter, but their distribution is consistent between species. The greatest density of CA terminals is found within the intermediolateral cell column (sympathetic lateral cell column or sympathetic preganglionic nucleus) of thoracic cord. Particularly dense accumulations also occur in the parasympathetic ganglion of the sacral cord, in the region surrounding the central canal (lamina X), surrounding motorneurons in the ventral horn (lamina IX) and in the superficial layers of the dorsal horn (laminae I and II). The more central parts of the dorsal horn (laminae III-VI) and the intermediate grey (lamina VII) generally contain fewer CA-fluorescent terminals.

Immunocytochemical studies employing antisera against the CA-synthesizing enzymes, tyrosine hydroxylase (TH) (Dietl et al., 1985; Pindzola et al., 1988), dopamine-ß-hydroxylase (DBH) (Fritschy and Grzanna, 1990; Glazer and Ross, 1980; Westlund et al., 1983, 1984), & phenylethanolamine-N-methyltransferase (PNMT) (Carlton et al., 1991; Hökfelt et al., 1974) or against the catecholamines themselves (Rajaofetra et al., 1992; Ridet et al., 1992; Wolters et al., 1989), have demonstrated that NA, DA, and AD all contribute to the spinal catecholaminergic innervation. This conclusion was also drawn after biochemical assays were made of spinal cord extracts (Andén, 1965; Fleetwood-Walker and Coote,

1981a,b; Karoum et al., 1980; Magnusson and Rosengren, 1963; Skagerberg et al., 1982; Zivin et al., 1975).

The relative contribution made by each CA varies considerably, however, with NA being found in much higher amounts than the other CA's (Fleetwood-Walker et al., 1981a,b; Karoum et al., 1980; Rajaofetra et al., 1992; Ridet et al., 1992; Skagerberg et al., 1982; Zivin et al., 1975). DA is present only in small amounts (about one-tenth that of NA) (Fleetwood-Walker et al., 1981a, 1981b; Karoum et al., 1980; Ridet et al., 1992; Skagerberg et al., 1982; Zivin et al., 1975) and AD is barely detectable (Carlton et al., 1991; Fleetwood-Walker et al., 1981a, 1981b; Hökfelt et al., 1974; Zivin et al., 1975).

Noradrenaline in the spinal cord.

Radioenzymic assays of microdissected regions of cat spinal grey matter (Fleetwood-Walker et al., 1981a, 1981b) have shown that NA levels are highest in the thoracic sympathetic nucleus (up to 13µg/g protein), and in the intermediate grey at lumbar regions (11µg/g protein). High amounts were also found in the ventral horn (about 9µg/g protein), but lower levels were detected in the dorsal horn (about 5µg/1g protein), even when the region being assayed contained laminae I and II. This data is similar to that reported by Zivin et al. (1975) in rabbits and rats, but contrasts with that of Skagerberg et al. (1982), who found more NA in the dorsal horn than in the ventral horn of rats.

Immunocytochemical studies employing antisera against the NA synthesizing enzyme dopamine-ß-hydroxylase (Fritschy and Grzanna, 1990; Glazer and Ross, 1980; Westlund et al., 1983, 1984) and NA itself (Rajaofetra et al., 1992) have indicated a distribution of NA fibres equivalent to that described for CA-histofluorescent structures (see earlier). This is only to be expected since NA is by far the most abundant CA in the spinal cord. The dopamine-ß-hydroxylase antibodies would also have labelled adrenergic axons, but the paucity of these structures in the spinal cord (see later) means that for all practical purposes they can be ignored.

Dopamine in the spinal cord.

The low levels of DA within the spinal cord meant that for many years it was regarded merely as a precursor for NA. The possibility of it acting as a neurotransmitter came from studies where lesioning of the noradrenergic cell groups in the brain did not concomitantly reduce spinal DA levels (Commissiong et al., 1978a,b; Fleetwood-Walker et al., 1981a,b; Karoum et al., 1980; Magnusson, 1973). An interesting observation was made by Commissiong et al. (1979) when calculating the ratio of NA to DA in various parts of the central nervous system. In cerebellum, where DA is still regarded as only a precursor to NA, the ratio was 47:1 (NA:DA). In the spinal cord the relative amounts of DA were much higher and the ratio was 15:1. The authors suggested that the

relatively high levels of DA in the spinal cord were not consistant with it being present simply as a precursor.

Skagerberg et al. (1982) have demonstrated the presence of specific dopaminergic nerve fibres within the spinal cord of rats. The authors found that they could achieve an almost complete depletion of spinal NA (94-99%) with 6-hydroxydopamine, but that DA levels were not significantly affected. Hence, when the spinal cord was reacted for CA-histofluorescence, most of the labelled axons were dopaminergic. The presence of dopaminergic fibres in the rat spinal cord has recently been confirmed by Ridet et al. (1992), using a specific antiserum to DA. Dopaminergic nerve fibres are found in the thoracic sympathetic nucleus, around the central canal, and in both dorsal and ventral horns, but the innervation to the ventral horn is weak. This distribution of DA reflects well the pattern observed in biochemical assays (Fleetwood-Walker et al., 1981a,b; Skagerberg et al., 1982; Zivin et al., 1985). Anatomical studies have shown that the dopaminergic and noradrenergic innervations to the dorsal horn are distributed differently (Ridet et al., 1992; Skagerberg et al., 1982): noradrenergic axons are found mainly in laminae I and II (see earlier), whereas DA-positive axons are concentrated in laminae III-V.

Adrenaline in the spinal cord.

The very low levels of AD within the spinal cord (Fleetwood-Walker et al., 1981a, 1981b; Zivin et al.,

1975) led to some doubt as to whether it was actually related to a nerve supply. This issue has now been resolved using antisera to PNMT (Carlton et al., 1991; Hökfelt et al., 1974). These workers demonstrated the presence of this enzyme within nerve endings innervating the thoracic sympathetic preganglionic nucleus and the region surrounding the central canal. The innervation to the remainder of the grey matter was sparse, but a few terminals were often found in laminae I and II (Carlton et al., 1991).

A large number of histofluorescence (Carlsson et al., 1962; Crutcher and Bingham, 1978; Dahlström and Fuxe, 1965; Fuxe, 1965; Lackner, 1980; Schroder and Skagerberg, 1982) and immunocytochemical (Fritschy and Grzanna, 1990; Glazer and Ross, 1980; Pindzola et al., 1988; Rajaofetra et al., 1992; Ridet et al., 1992; Westlund et al., 1983, 1984) studies have reported that the only CA-containing structures present in the spinal cord are nerve fibres and terminals.

However, in 1983, Singhaniyom et al. reported the presence of previously undescribed CA-fluorescent neuronal perikarya within the white and grey matter of the rat upper cervical spinal cord (segments C1 and C2). Cell bodies within the white matter were found to be anatomically continuous with the catecholaminergic cells of the A1 medullary group, while those cells found within the cervical grey matter joined the A2 group in the lower

medulla. The authors counted about 300 CA-containing perikarya in C1 and C2 on each side of the cord. The presence of these cells has been confirmed by a group of French anatomists (Dietl et al., 1985; Mouchet et al., 1986), using immunostaining methods, and they are generally regarded to represent caudal extentions of the medullary neuron groups. Using antibodies to TH, DBH and PNMT as comparative markers (Mouchet et al., 1986), it was shown that the group of cells in the white matter is noradrenergic, while the cluster in the grey matter contains both noradrenergic and dopaminergic cells in roughly equal numbers.

These later studies (Dietl et al., 1985; Mouchet et al., 1986) were not confined to the cervical cord, and investigated the possibility that CA-containing cell bodies occur in other regions of the spinal cord. Thoracic and lumbar regions were only very occasionally observed to possess CA-containing perikarya, but a prominant population of cells was found in the first sacral segments. Because these cells were positive for TH, but not DBH, the authors concluded that they were dopaminergic. However, it has recently been shown that there are neurons in the brain which contain TH but not CA (Vincent and Hope, 1990). Since these sacral spinal cord cells also contain TH, but do not display CA-histofluorescence (Carlsson et al., 1964; Dahlström and Fuxe, 1965; Schroder and Skagerberg, 1985), it is probable that they too are non-catecholaminergic.

(e). **Brainstem origins of spinal cord catecholamines.**

Early evidence that the catecholaminergic innervation of the spinal cord arose from cell bodies located in supraspinal sites came from observations that CA-histofluorescence (Carlsson et al., 1962; Dahlström and Fuxe, 1965), NA levels (Magnusson and Rosengren, 1963) and dopamine-ß-hydroxylase activity (Glazer and Ross, 1980) fell dramatically or completely disappeared following complete transection of the cord. Earlier, Dahlström and Fuxe (1964) had mapped the distribution of CA-containing perikarya in the rat brainstem, and it was proposed that the spinal CA-containing axons originate from one or more of these nuclei (Dahlström and Fuxe, 1965).

A variety of methods have been used to identify which CA-containing cell groups project to the spinal cord. These include stereotaxic lesioning of a CA nucleus in the brain, followed by biochemical or histochemical analysis of the spinal cord CA content. Conclusions from these studies were based upon any changes in the staining pattern being observed after the lesion. A second approach used was the retrograde labelling of CA nuclei in the brain following the application of a tracer substance into the spinal cord. Anterograde tracing with tritiated amino acids has also been been used and, more recently, with *Phaseolus vulgaris* leucoagglutinin.

Using these techniques, a number of NA-containing cell groups have been proposed to project to the spinal cord.

These are the the nucleus locus coeruleus (A6 cell group), the subcoeruleus, parabrachial and Kölliker-Fuse nuclei (A7 cell group) and the A5 group. Spinal projections from the adrenergic C1 group and the DA-containing A11 group have also been reported. The evidence suggesting projections to the spinal cord from these cell groups will now be assessed.

1. A6 cell group (locus coeruleus, LC).

The LC cell group has been the most extensively studied brainstem CA-containing cell group, and it has been demonstrated conclusively to send a major noradrenergic projection to the spinal cord in a number of species.

Many authors have found that injecting HRP into the cervical, thoracic and lumbosacral segments of the spinal cord will produce retrograde labelling of LC neurons. These studies have been performed in the rat (Ader et al., 1979; Basbaum and Fields, 1979), cat (Basbaum and Fields, 1979; Hancock and Fougerousse, 1976; Hayes and Rustioni, 1981; Kuypers and Maisky, 1975), monkey (Hancock and Fougerousse, 1976; Kneisley et al., 1978) and North American opossum (Crutcher et al., 1978; Martin et al., 1979a,b). Furthermore, double labelling studies, in which the retograde transport is combined with CA-histofluorescence (Blessing et al., 1981; Martin et al., 1982; Stevens et al., 1982), TH- or DBH-immunostaining (Clark and Proudfit, 1991a; Clark et al., 1991; Fritschy and Grzanna, 1990; Pindzola et al., 1988; Westlund et

al., 1983, 1984), or monoamine oxidase staining (Satoh et al., 1977) have shown that the great majority of the retrogradely labelled LC cells also contain NA. Furthermore, most are located in the ventral part of the nucleus.

Westlund et al. (1981, 1983, 1984) have further examined the coerulospinal projection in rats and monkeys using the retrograde transport of antibody to DBH. This method selectively labels neurons using NA and AD as neurotransmitters. Results from these experiments have showed that the LC contains 29% (monkey) & 41% (rat) of the NA-containing cells that innervate the spinal cord in these species. Fritschy and Grzanna (1990) produced a slightly lower estimate for the rat (31%) when combining the retrograde transport of True Blue with DBH-immunostaining. Results from double labelling studies also support a major descending noradrenergic projection from the LC in the North American opossum (Martin et al., 1982; Pindzola et al., 1988). However, in the rabbit (Blessing et al., 1981) and the cat (Stevens et al., 1982), the contribution made by the LC to the spinal noradrenergic innervation is only minor.

In agreement with these findings, electrolytic lesioning of the A6 group in the rat has been reported to produce a marked reduction in CA-histofluorescence (Commissiong et al., 1978b; Nygren alnd Olson, 1977), NA levels (Ader et al., 1979; Karoum et al., 1980) and DBH levels (Clark and Proudfit, 1991a; Ross and Reis, 1974)

in the spinal cord. A major coerulospinal projection has also been reported in the cat from the results obtained in LC lesion experiments (Fleetwood-Walker and Coote, 1981a). However, this is inconsistent with the findings of Stevens et al. (1982), in retrograde tracing studies. One possible explanation of this discrepancy is that the ablations performed by Fleetwood-Walker and Coote (1981a) may have damaged descending axons from other noradrenergic cell groups, which pass through or close by the LC.

A number of recent studies, in two different substrains of Sprague-Dawley rat, have used the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHAL) to examine LC axon terminations in the spinal cord (Clark and Proudfit, 1991a, 1992; Fritschy and Grzanna, 1990). In rats supplied by SASCO, the LC was found to primarily innervate the ventral horn (laminae VIII and IX) and the intermediate zone (lamina VII) (Clark and Proudfit, 1991a), with only sparse labelling being produced in the dorsal horn. In contrast, in rats supplied by HARLAN (Clark and Proudfit, 1992; Fritschy and Grzanna, 1990), the LC was found to terminate most heavily in the dorsal horn (particularly laminae I-IV), with only a few labelled axons been seen in ventral horn and intermediate zone. Double-labelling studies with antisera to DBH confirmed that the majority of PHAL-labelled LC axons were noradrenergic (Clark and Proudfit, 1991a, 1992; Fritschy and Grzanna, 1990). It was thus concluded that the two substrains of rat are

fundamentally different in the organization of their descending noradrenergic projections. These differences were confirmed in retrograde tracing studies in which injections of Fluoro-Gold (Clark et al., 1991) or fluorescent latex beads (Sluka and Westlund, 1992) were restricted to the dorsal or ventral horns.

The innervation to the ventral horn and lamina VII by LC neurons in SASCO rats appears to be similar to the pattern of innervation in several other rat substrains and other mammalian species. For instance, in rats, bilateral LC lesions produce an almost complete loss of CA-histofluorescent terminals in the ventral horn and intermediate grey, but result in only a small reduction in the dorsal horn (Commissiong et al., 1978b; Nygren and Olson, 1977). Comparable lesions in cats have been reported to produce a similar pattern of CA depletion (Fleetwood-Walker et al., 1981a). Furthermore, in anterograde tracing experiments, where tritiated amino-acids were injected into dorsolateral pons of monkeys (Westlund and Coulter, 1980) and the North American opossum (Martin et al., 1979a,b), placements which included the LC, terminal labelling was most heavy over the ventral horn and lamina VII.

In all rat substrains examined (Clark and Proudfit, 1991a; Commissiong et al., 1978b; Fritschy and Grzanna, 1990; Nygren and Olson, 1977;) & in monkey (Westlund and Coulter, 1980) there appears to be little contribution from the LC to the NA innervation of the thoracic

sympathetic lateral cell column. However, moderate innervations from the LC to this region have been reported in the cat (Fleetwood-Walker and Coote, 1981a), & North American opossum (Martin et al., 1979b). Anterograde tracing studies have shown a heavy projection from the LC to the sacral parasympathetic nucleus in the monkey (Westlund and Coulter, 1980).

2. A1 and A2 cell groups.

These two cell groups were the first that were proposed to project to the spinal cord. Dahlström and Fuxe (1965) observed, in rats, that neurons in these regions showed greatly enhanced CA-fluorescence following spinal cord transection. These changes were explained as an accumulation of CA within the cell bodies as a consequence of axonal severing. However, this study gave no indication of which spinal cord regions receive projections from these groups.

The lesion studies of Fleetwood-Walker and Coote (1981a), in cats, have shown that there is a major projection from the A1 and and a minor projection from the A2 cell groups to the thoracic intermediolateral cell column. Following bilateral lesions of the A1 and A2 neurons there was a 77% and 18% reduction respectively in NA levels within this region. In addition, electrophysiological studies from the same laboratory (Fleetwood-Walker et al., 1983) have found cells within the area of the A1 cell group which can be antidromically activated by stimulation of their axons within the

thoracic cord. Studies in which retrograde tracers were injected into the thoracic cord, in an attempt to label the CA-containing cells in the medulla, have produced variable results. Some authors favour the idea of a descending projection. In these studies, performed in rat (Jeske and Mckenna, 1992; Ross et al., 1981; Satoh et al., 1977), rabbit (Blessing et al., 1981), chick (Smolen et al., 1979), primate (Carlton et al., 1991) and North American opossum (Pindzola et al., 1988), large numbers of the A1 CA-containing cells were retrogradely labelled. These observations, when combined with data from lesioning studies (Fleetwood-Walker and Coote, 1981a), strongly suggest that there is a major descending CA pathway from the A1 group to the thoracic sympathetic nucleus. This projection is considered to arise specifically from adrenergic neurons in the rostral pole of the nucleus (i.e. the C1 region) (Blessing et al., 1981; Carlton et al., 1991; Ross et al., 1981). Jeske and McKenna (1992) have estimated that at least 81% of PNMT-positive C1 neurons of rat project to the thoracic cord. CA-containing cells in the A2-C2 group were never retrogradely labelled after spinal cord tracer injections (Blessing et al., 1981; Carlton et al., 1991; Ross et al., 1981; Smolen et al., 1979), although a small number might have been anticipated on the basis of results from lesioning experiments (Fleetwood-Walker and Coote, 1981a).

Lesion studies have provided no evidence for a CA

projection to the dorsal and ventral horns from the A1-C1 or A2-C2 groups (Fleetwood-Walker and Coote, 1981a). However, following HRP implants into the DLF at lumbosacral levels, a small number of PNMT-containing neurons in the C1 region (1% or less) were double labelled (Carlton et al., 1991).

In marked contrast to the results obtained in these experiments, a number of retrograde labelling studies in rats have suggested that these medullary cell groups make no contribution at all to the spinal cord CA content, even in the thoracic segments (Fritschy and Grzanna, 1990; Westlund et al., 1981, 1983, 1984). However, the reasons for this discrepancy are not apparent. In most studies (Blessing et al., 1981; Fritschy and Grzanna, 1990; Jeske & McKenna, 1992; Smolen et al., 1979; Westlund et al., 1981, 1983, 1984) non-catecholaminergic neurons in both the A1 and A2 cell groups were observed to project to the spinal cord.

3. A4 cell group.

The contribution made by this nucleus to the spinal cord CA content is extremely small. Following injection of HRP (Blessing et al., 1981) or fluorophores (Sluka and Westlund, 1992) into the spinal cord only an occasional CA-containing cell was labelled in the A4 group.

4. A5 cell group.

There is abundant anatomical evidence showing that

noradrenergic neurons located in the A5 group project to the spinal cord.

Anterograde transport of tritiated amino-acids (Loewy et al., 1979) or *Phaseolus vulgaris* leucoagglutinin (PHAL) (Clark and Proudfit, 1993), injected into the region of the A5 cell group in rats, revealed a dense projection to the thoracic intermediolateral cell column. Double-labelling studies with an antiserum to DBH confirmed that the majority of these axons were noradrenergic (Clark and Proudfit, 1993). Furthermore, this labelling was abolished by pretreatment with 6-OHDA (Loewy et al., 1979). CA-fluorescent neurons in the A5 region have also been retrogradely labelled following HRP injections into a region of thoracic spinal cord which included the lateral horn (Blessing et al., 1981; Byrum et al., 1984; Fritschy and Grzanna, 1990; Loewy et al., 1979; Satoh et al., 1977; Westlund et al., 1983). Byrum et al. (1984) found that mid-thoracic injections of HRP labelled up to 93% of NA-containing cells in the A5 area, and that NA-containing cells constituted at least 90% of the spinally projecting cells present.

Retrograde tracing studies have also revealed a major descending projection from the A5 cells to the cervical and lumbosacral spinal cord, at least in the rat (Clark and Proudfit, 1991a; Clark et al., 1991; Fritschy and Grzanna, 1990; Satoh et al., 1977; Westlund et al., 1983) and monkey (Westlund et al., 1984). Westlund's group, using retrograde tracing with DBH-antibodies, proposed

that the A5 group contains 8% of the total number of noradrenergic neurons projecting to the spinal cord in the rat, and 11% of the total in the monkey. Fritschy and Grzanna (1990) produced a larger estimate for the rat (20-30%), when combining HRP transport with DBH-immunostaining. Anterograde tracing studies in the rat, with PHAL (Clark and Proudfit, 1993), showed that A5 neurons project mainly to the deep dorsal horn (laminae IV-VI) and the intermediate zone (lamina VII), and double-labelling studies confirmed that most of these axons were noradrenergic.

In contrast to these studies, Fleetwood-Walker and Coote (1981a), in a lesioning study, found no evidence for a descending CA projection from the A5 group to the cat spinal cord. Basbaum and Fields (1979) have retrogradely labelled A5 neurons in the cat with HRP from lumbar injections, but unfortunately they did not attempt to double-label these cells for NA. It may be, however, that in cats the A5 projection to the spinal cord does not contain CA. Similarly, Pindzola et al. (1988) found few, if any, CA cells in this region double-labelled, following large injections of fluorophores into the spinal cord of the North American opossum.

5. A7 cell group.

The pontine A7 cell group is probably the source of more descending CA-containing neurons than any other brainstem group, and in all species. Anatomically, it is diffusely organized into a number of smaller subgroups.

For the purposes of this account, the A7 group is considered to comprise the nucleus subcoeruleus (SC), the medial and lateral parabrachial nuclei (PBN) and the Kölliker-Fuse nucleus (KFN), as first proposed by Dahlström and Fuxe (1964).

Although fibre degeneration has been observed in the cat spinal cord, as a result of a small electrolytic lesion in the PBN (Takeuchi et al., 1980), most of the evidence proposing a major spinal projection from this region of the brain comes from retrograde axonal tracing studies. Investigations in the rat (Basbaum and Fields, 1979), cat (Basbaum and Fields, 1979; Hancock and Fougerousse, 1976; Hayes and Rustioni, 1981; Kuypers and Maiskey, 1975; Takeuchi et al., 1980), monkey (Hancock and Fougerousse, 1976; Kneisley et al., 1978) and North American opossum (Martin et al., 1979a,b) have observed labelled cells in the A7 region following HRP injections at all spinal cord levels. Moreover, the specific uptake and transport of antibody to DBH (Westlund et al., 1981, 1983, 1984), and double-labelling methods (Blessing et al., 1981; Clark and Proudfit, 1991a,b; Clark et al., 1991; Fritschy and Grzanna, 1990; Martin et al., 1982; Pindzola et al., 1988; Satoh et al., 1977; Sluka and Westlund, 1992; Stevens et al., 1982; Westlund et al., 1983, 1984), have demonstrated that most of these projections are nordrenergic.

The contribution made by each subgroup of the A7 region to the spinal cord innervation varies

considerably, however, and is also species dependant. In the rat, Westlund et al. (1983) have shown that 45% of the NA-containing neurons projecting to the spinal cord originate in the SC. Lesser projections arise from the KFN (5%) and PBN (1%). Their later study in the monkey (Westlund et al., 1984) produced very similar results, with the SC providing 49% of of spinally projecting noradrenergic neurons, the KFN 9% and the PBN 1%. Similar proportions have also been reported in the North American opossum (Martin et al., 1982; Pindzola et al., 1988). However, in cats the relative contributions are quite different (Stevens et al., 1982), since the KFN is the principal source of descending NA-containing cells in this species. Very few descending cells are located in the SC-PBN (or in the A6 group).

Anterograde tracing experiments with PHAL, in SASCO rats, have shown that the SC terminates mainly in the ventral horn (Clark and Proudfit, 1991a), whereas other subnuclei of the A7 group terminate mainly in the dorsal horn, particularly in laminae I-IV (Clark and Proudfit, 1991b). Double-labelling studies, with an antiserum to DBH, confirmed that nearly all of the PHAL-labelled axons also contained NA. Retrograde labelling experiments, in which injections of a tracer substance were confined to a single dorsal or ventral horn, have confirmed these findings (Clark et al., 1991; Sluka and Westlund, 1991). In HARLAN rats, the opposite situation exists (Clark et al., 1991; Sluka and Westlund, 1992). In this substrain, the SC projects mainly to the dorsal horn, while the

other A7 regions (PBN and KFN) project mainly to the ventral horn. In the monkey (Westlund and Coulter, 1980), injections of [^{3}H] amino acids into the SC/PBN region produced heavy labelling in the thoracic sympathetic cell column, the ventral horn, lamina X and lamina I.

6. A8-A14 cell groups.

Direct hypothalamic projections to the spinal cord have been established with the HRP method in several mammals, including rat (Basbaum and Fields, 1979; Hancock, 1976; Hosoya, 1980), rabbit (Blessing and Chalmers, 1979), opossum (Crutcher et al., 1978), cat (Basbaum and Fields, 1979; Kuypers and Maisky, 1975) and monkey (Kneisley et al., 1978). More specifically, labelled cells were found in the A13 dopamine cell group of the dorsal hypothalamus, the A14 cell group of the anterior hypothalamus (periventricular grey) and the posterior hypothalamic component of the A11 cell group.

However, when retrograde transport studies are combined with CA-histofluorescence, a much more restricted distribution for the descending dopaminergic cells is observed (Björklund and Skagerberg, 1979; Hökfelt et al., 1979; Skagerberg et al., 1982; Skagerberg and Lindvall, 1985). These investigations, which were performed in the rat, found large numbers of retrogradely labelled DA-containing cells in the diencephalic A11 cell group. This group is now thought to be the only

dopaminergic cell group which projects to the spinal cord in the rat, since no retrogradely labelled, DA-positive, cells were found in the A8, A9 or A10 cell groups. Furthermore, no labelled cells were found in the A13 group, although a single labelled periventricular DA cell was reported in the A14 cell group. The proportion of A11 DA-containing cells which project to the spinal cord has been found to vary from one study to another. Björklund and Skagerberg (1979) reported that almost all of the A11 DA cells project to the cord. However, Skagerberg and Lindvall (1985) found that only 20-40% of these cells descended.

In accord with these anatomical findings, physiological data also suggests that the spinal DA innervation in rats is derived solely from the diencephalic A11 group. For instance, Fleetwood-Walker et al. (1988) found that electrical stimulation in the region of the A11, but not the A9, cell group inhibited dorsal horn neurons in the rat. Similarly, Duggal and Barasi (1985) failed to demonstrate any stimulus evoked action from the A9 group in behavioural studies. A contribution to the spinal DA innervation from the A9 region was suggested by Commissiong et al. (1979), who observed a small decrease in spinal DA levels following lesions of the nigral region. In addition, Segal and Sandberg (1977) found that stimulation in the region of the substantia nigra increased the latency to perceive noxious heat. However, it has been have suggested that the antinociceptive effect cited by these authors may

have been due to current spread outside the region of the nigra (Duggal and Barasi, 1985). Furthermore, Hokfelt et al. (1979) have suggested that the lesions made by Commissiong et al. (1979) could have interfered with descending projections from the more rostrally located A11 DA cells.

Thus, in the rat the majority of evidence suggests that the spinal DA-containing axons arise exclusively from the A11 cell group. This is also true for the North American opossum (Pindzola et al., 1988). However, in the rabbit the spinal DA innervation has been reported to arise from the A13 cells in the dorsal hypothalamus (Blessing and Chalmers, 1979). Thus, as with the NA-containing cell groups of the pons, there may be species differences in the contribution made by each dopaminergic nucleus to the spinal CA innervation.

(f). **Routes of descent of axons.**

In their pioneering study, Carlsson et al. (1964) obtained evidence that CA fibres descend in the lateral and ventral funiculi to terminate in the grey matter. Following treatment with nialamide, a monoamine oxidase inhibitor, large numbers of descending fluorescent axons appeared in these regions. A comparable approach was used by Fleetwood-Walker and Coote (1981b), who observed an accumulation of NA in the lateral and ventral funiculi rostral to a cord transection at T3.

Dahlström and Fuxe (1965) have proposed that the

descending CA axons are organized into two separate systems. A large system runs in the ventral and ventrolateral funiculi to terminate in the ventral horn, while a separate, smaller system descends in the dorsolateral funiculus (DLF) and serves the dorsal horn and thoracic intermediolateral cell column. This conclusion was also drawn by Martin et al. (1979a,b), following injections of [^{3}H]-leucine into the lateral pons.

Anterograde tracing studies in SASCO Sprague-Dawley rats (Clark and Proudfit, 1991a), and in monkeys (Westlund and Coulter, 1980), have shown that descending noradrenergic axons from the locus coeruleus (A6 group) and the nucleus subcoeruleus course through the ventral and ventrolateral funiculi before terminating in the ventral horn. Very few axons were observed in the DLF. Further, Nygren and Olson (1977) found that lesioning the LC in rats (substrain not specified) abolished CA-fluorescence in the ventral quadrant (VQ), while CA-axons in the DLF were unaffected. Mokha et al. (1986) have proposed that the ventral quadrant also provides the only route of descent for LC axons in the cat. These authors found that only lesions which involved the VQ blocked the LC-induced inhibition of dorsal horn neurons. Recently, Fritschy and Grzanna (1990) and Clark and Proudfit (1992) have challenged the classical view that the descending LC axons course exclusively through the white matter. In these studies, performed in HARLAN rats, very few fibres were labelled in the VQ and DLF after injections of PHAL

into the LC. In contrast, large numbers of rostrocaudally oriented axons were found in laminae I and II of the dorsal horn, most of which also contained DBH. Furthermore, following complete destruction of the LC using the selective neurotoxin DSP-4 (Fritschy and Grzanna, 1990), the extensive plexus of axons in laminae I and II disappeared. On this basis it was proposed that the superficial layers of the dorsal horn provide the major route of descent for coerulospinal fibres in HARLAN Sprague-Dawley rats.

Noradrenergic axons from the KFN and PBN (A7 subgroups) (Clark and Proudfit, 1991b) and the A5 group (Clark and Proudfit, 1993; Loewy et al., 1979), and dopaminergic axons from the A11 cell group (Fleetwood-Walker et al., 1981b; Ridet et al., 1992; Skagerberg et al., 1982) descend mainly in the dorsolateral funiculi.

An important consideration of anatomical studies is whether the descending CA-containing neurons project ipsilaterally or cross to the other side of the cord before termination. Although the results from lesioning studies are inconclusive (Ader et al., 1979; Karoum et al., 1980; Ross and Reis, 1974), retrograde tracing studies (Clark et al., 1992; Fritschy and Grzanna, 1990; Sluka and Westlund, 1992; Stevens et al., 1982), and anterograde tracing studies (Clark and Proudfit, 1991a,b, 1993; Fritschy and Grzanna, 1990) have demonstrated that NA-containing axons from the A5, A6 and A7 cell groups project bilaterally to the spinal cord in a number of

species. However, the ipsilateral side was more heavily labelled than the contralateral side in these studies, indicating a predominantly uncrossed pathway. A mostly ipsilateral trajectory has also been reported for the adrenergic C1 cells of the medulla (Jeske and McKenna, 1992), although the A11 dopamine pathway has been proposed to descend completely uncrossed (Skagerberg et al., 1982).

(g). **Other possible origins of spinal cord catecholamine.**

Transection studies have confirmed that most, if not all, of the CA-containing nerve terminals in the spinal cord originate from higher centres (Carlsson et al., 1962; Dahlström and Fuxe, 1965; Glazer and Ross, 1980; Magnusson and Rosengren, 1963), possibly including the CA-containing perikarya of the upper cervical cord (Mouchet et al., 1986; Singhaniyom et al., 1983).

However, Price and Mudge (1983) have reported that a small number, about 1%, of rat lumbar dorsal root ganglion cells display immunoreactivity to TH. These cells did not contain DBH, and were suggested to be dopaminergic. No TH-positive sensory neurons were present rostral to L5. These findings have been confirmed by Kummer et al. (1988) in the guinea-pig, although in this species, TH-immunoreactive perikarya were additionally found in segments L1-L4 and were generally more numerous than in the rat. These dorsal root ganglion cells did not, however, exhibit glyoxylic acid- or faglu-induced CA-fluorescence (Kummer et al.,

1988; Price and Mudge, 1983). Furthermore, HPLC analysis failed to detect any DA in the ganglia (Kummer et al., 1988). Based on these findings, it was suggested that TH-immunoreactive sensory neurons do not synthesize DA (Kummer et al., 1988). Hence, it is unlikely that they make any contribution to the catecholaminergic innervation of the spinal cord.

Similarly, the TH-immunoreactive neurons found in the sacral segments of the spinal cord do not contain DBH (Mouchet et al., 1986): nor do they display CA-histofluorescence (Carlsson et al., 1962; Dahlström and Fuxe, 1965; Schroder and Skagerberg, 1985). Hence, it is likely that they too are non-dopaminergic, and that they make no contribution to the spinal cord CA content.

Thus, with the possible exception of a small contribution from the CA-containing perikarya in C1 and C2, the spinal cord CA content is otherwise derived exclusively from supraspinal sites.

3. ACTIONS OF CATECHOLAMINES IN THE SPINAL DORSAL HORN.

Descending CA projections have been shown to regulate sensory, motor and autonomic activity in the spinal cord. The autonomic effects are mediated by CA terminals in the preganglionic nuclei of thoracic and sacral segments, while the motor effects result from actions on the motoneurons of the ventral horn.

Of particular significance to this review are the

actions that CA's exert upon sensory transmission, effects which are mediated by CA-containing terminals within the dorsal horn. The following sub-sections review the behavioural and electrophysiological data suggesting that these pathways inhibit sensory transmission, an effect which has received particular attention in relation to nociception.

(a). **Results from behavioural studies.**

The technique of intrathecal (i.t.) administration of drugs via chronically implanted spinal catheters has proven to be very useful for pharmacological studies of the spinal analgesic actions of NA and DA.

1. Noradrenaline.

I.t. administration of NA into the lumbar sub-arachnoid space of rats (Kuraishi et al., 1979b, 1985b; Reddy and Yaksh, 1980), cats (Reddy and Yaksh, 1980), mice (Hylden and Wilcox, 1983) and primates (Yaksh and Reddy, 1981) has been shown to produce a rapid, behaviourally defined analgesia in thermal, mechanical and chemical algesic tests. Kuraishi et al. (1985b) have examined the antinociceptive effect of NA using three different assays (Tail-pinch, Hot-plate and Tail-flick). In the tail-pinch method, forceps were applied to the tail and the latency of biting responses to the forceps was measured. In the hot-plate method, rats were placed onto a copper plate (55°C) and the latency of licking either hind-paw was measured. In the tail-flick method,

the latency of tail flexion in response to radiant heat was measured. NA produced a dose-dependant increase in the response times in all three tests (analgesia), but the effect was most prominant upon the mechanical nociceptive pathways (tail-pinch assay). These authors (Kuraishi et al., 1985b) went on to compare the efficacy of NA in these tests with that of morphine, met-enkephalin and serotonin. NA was found to be the most potent drug at producing analgesia. For example, in the hot-plate, tail-flick and tail-pinch assays, NA was 40, 200, and 700 times more potent respectively than serotonin.

The antinociceptive actions of i.t. NA can be mimicked by the selective α_2-adrenoceptor agonist clonidine (Hylden and Wilcox, 1983; Tjolsen et al., 1990) but not by the ß-agonist isoproterenol (Reddy and Yaksh, 1980). Phenylephrine, a selective α_1-agonist, does produce analgesia but it is ten times less potent than NA (Hylden and Wilcox, 1983; Reddy and Yaksh, 1980). In addition, the antinociceptive actions of NA can be reversed, in a dose-dependant manner, by i.t. administration of the selective α_2-antagonist yohimbine (Hylden and Wilcox, 1983) but not by the ß-antagonist propranolol (Hylden and Wilcox, 1983; Reddy and Yaksh, 1980). The selective α_1-antagonist prazosin is also effective, but only at very high doses (Hylden and Wilcox, 1983). These findings suggest that NA, released from the descending axons of brainstem neurons, acts preferentially upon adrenoceptors

of the α_2 subtype. The indirectly acting adrenergic agonist cocaine has also been reported to produce analgesia when administered i.t. (Roerig et al., 1992). In this study, cocaine inhibited the irritative behavioural responses elicited in mice by i.t. substance P. This effect was antagonised by low doses of the α_2-antagonists yohimbine and idazoxan, but not by prazosin (an α_1-antagonist).

Agents which produce an accumulation of NA at the synapse have been shown to markedly potentiate NA analgesia. For instance, pretreatment with the NA uptake blockers protryptyline (Reddy and Yaksh, 1980) and desipramine (Sawynok and Reid, 1992) produced a massive increase in the intensity and duration of the analgesia elicited by i.t. NA. Administration of Lilley 51641 (a monoamine oxidase inhibitor) also had the same effect (Reddy and Yaksh, 1980).

These descending noradrenergic pathways have been suggested to mediate, at least partly, the analgesia produced by supraspinally administered morphine. For instance, i.t. injection of phentolamine and phenoxybenzamine reversed the antinociception produced when morphine was injected into the nucleus reticularis gigantocellularis (Kuraishi et al., 1979a) and periaqueductal grey (Yaksh, 1979) of the rat. Furthermore, i.t. injection of phentolamine blocked the analgesia produced by electrically stimulating the nuclei reticularis gigantocellularis and raphe magnus in the same species (Hammond and Yaksh, 1984).

Hylden and co-workers (Hylden and Wilcox, 1983; Roerig et al., 1992) have investigated possible interactions between α_2-adrenergic and opioid systems in the spinal cord of mice. In the substance P behavioural assay, i.t. co-administration of morphine (or the selective δ-opioid agonist D-Pen-D-Pen-enkephalin) potentiated the analgesic actions of NA, clonidine and cocaine, thus indicating a spinal synergism between the two transmitter systems. In contrast, there was no synergism between the μ-opioid agonist [Tyr-D-ala-NMe-Phe-Gly(ol)] and α-adrenergic drugs. These findings indicate that spinal δ-opioid receptors are involved in the opioid-NA interaction. This spinal synergism has clinical importance, since epidurally administered clonidine has been reported to decrease the dose of intravenous morphine required for postsurgical pain relief (Eisenach et al., 1989).

Evidence has been presented, in rats, that the descending noradrenergic system is tonically active (Jones and Gebhart, 1986; Sagan and Proudfit, 1984). These authors found that i.t. injections of yohimbine produced a dose dependant decrease in nociceptive threshold in both tail-flick and hot-plate assays (hyperalgesia). This conclusion has been challenged by Tjolsen et al. (1992), who performed similar experiments. These authors found yohimbine to be inactive in the hot-plate assay and, although a reduction in tail-flick latency was observed, it could be accounted for by the fall in skin temperature which accompanied this effect.

In a recent study, Jones (1992) showed that the heat induced expression of fos-immunoreactivity in dorsal horn neurons, was inhibited by i.t. NA.

In behavioural studies, it is critical to establish that the actions of the α-adrenergic drugs are restricted to the site of their i.t. injection (i.e. the lumbar spinal cord). The rapid onset of their action (within 5 minutes) suggests that this is the case (Hylden and Wilcox, 1983; Kuraishi et al., 1979, 1985; Reddy and Yaksh, 1980; Tjolsen et al., 1992). Furthermore, the cutaneous area of analgesia that results from such injections is restricted to the caudal dermatomes of the animal (Reddy and Yaksh, 1980). In addition, animals in these studies did not show any signs of motor weakness or sedation, and continued normal exploratory behaviour during periods of prolonged analgesia (Hylden and Wilcox, 1983; Kuraishi et al., 1979, 1985; Reddy and Yaksh, 1980). This suggests that α_2-mediated antinociception, produced by i.t. NA, results from a direct action upon dorsal horn neurons, and does not involve an action upon motoneurons or the brain. Furthermore, these effects are not a consequence of degeneration resulting from vasoconstriction and ischaemia, since papaverine, a vasodilator, does not antagonize the analgesic effect of NA (Reddy and Yaksh, 1980).

2. Dopamine.

In similar way to adrenergic agonists, i.t. administration of the D_2 DA-receptor agonist apomorphine

produced a dose-dependant increase of the hot-plate response latency in rats (Jensen and Yaksh, 1984). An inhibitory effect was also observed on the acetic acid-induced writhing response. Furthermore, i.t. DA has been reported to prolong the tail-flick response latency in rats, an effect which was attributed to activation of a D_2 receptor, since it was blocked by sulpiride (a D_2 antagonist) but not by SCH 23390 (a D_1 antagonist) (Liu et al., 1992).

However, in marked contrast to the results of Liu et al. (1992), other authors found that apomorphine and DA did not influence tail-flick latency even at high doses (Jensen and Smith, 1983; Jensen et al., 1984; Jensen and Yaksh, 1984). Following total spinal cord transection or bilateral lesioning of the DLF, however, this assay was rendered sensitive to inhibition by these agents, suggesting that supraspinal pathways exert an inhibitory action upon the DA-induced depression in this reflex. It has been proposed that the systems involved may be noradrenergic or serotoninergic since i.t. pretreatment with phentolamine or methysergide (a serotonin antagonist) rendered the tail-flick sensitive to inhibition by apomorphine (Jensen and Yaksh, 1984). The mechanism behind this inhibitory action is not clear. However, it has been suggested that facilitatory influences exerted on the motoneurons (Bell and Matsumiya, 1981; Strahlendorf et al., 1980; White and Neuman, 1980) by tonically active descending monoaminergic systems

could physiologically antagonise the attenuation produced by DA in the dorsal horn. Therefore, spinal transection, DLF lesioning and treatment with monoaminergic antagonists may remove this facilitatory tone, rendering the reflex pathway sensitive to modulation by DA.

The reason for this disparity, between different rat strains, in the sensitivity of the tail-flick assay to modulation by DA agonists is unknown, but strain differences in the distribution of descending monoaminenergic axons is one possibility (Clark and Proudfit, 1991a,b, 1992; Clark et al., 1991; Sluka and Westlund, 1992). Alternatively, Liu et al. (1992) injected their rats with barbiturates prior to testing, which could have rendered the assay sensitive to modulation.

(b). **Results from ionophoretic studies.**

1. Noradrenaline.

Many groups have investigated the actions of ionophoretically applied NA upon dorsal horn neurons which were activated by peripheral stimulation. In general, the predominant effect observed is inhibition; NA depressing the activity generated in dorsal horn neurons by cutaneous stimuli (Belcher et al., 1978; Davies and Quinlan, 1985; Engberg and Ryall, 1966; Fleetwood-Walker et al., 1985; Headley et al., 1978; Howe and Zieglgänsberger, 1987). Excitatory effects are usually not seen at all, or are greatly outnumbered by

cells responding in an inhibitory manner. A small number of cells are unaffected by ionophoretically applied NA (Engberg and Ryall, 1966; Fleetwood-Walker et al., 1985; Howe and Zieglgänsberger, 1987) or respond in a complex way (Howe and Zieglgänsberger, 1987). Complex effects consist of an initial period of inhibition followed by excitation. These ionophoretic studies were performed in cats (Belcher et al., 1978; Davies and Quinlan, 1985; Engberg and Ryall, 1966; Fleetwood-Walker et al., 1985; Headley et al., 1978), rats (Howe and Zieglgänsberger) and rabbits (Satoh et al., 1979).

An important conclusion of many ionophoresis investigations is that the regulatory effect upon sensory transmission is selective for, or more pronounced upon, activity generated by noxious stimuli (Belcher et al., 1978; Davies and Quinlan, 1985; Fleetwood-Walker et al., 1985; Headley et al., 1978). For example, Fleetwood-Walker et al. (1985) found that NA produced a potent, selective inhibition of the nociceptive responses (noxious heat or pinch) in 40/44 multireceptive spinocervical tract cells. Little or no effect was observed on the responses to innocuous brush. In addition, NA had no effect on the majority of cells (8/11) that responded only to low threshold stimuli. In accordance with these findings, Belcher et al. (1978) have reported that NA ionophoresed in the vicinity of low threshold cells produces no effect, yet it reduces the activity evoked by noxious heat in most (82%) nociceptive

cells tested.

In contrast to these findings, Satoh et al. (1979) found that NA inhibited both nociceptive and non-nociceptive responses in rabbit wide dynamic range neurons. Fleetwood-Walker et al. (1985) argued that this non-selective action was a consequence of the high ejection currents (up to 100nA) used by these authors. However, Howe and Zieglgänsberger (1987), found no selectivity in the action of NA at ionophoretic currents similar to those used by Fleetwood-Walker et al. (1985).

Headley et al. (1978) have recorded from multireceptive neurons in laminae IV and V, and compared the effects of ionophoresing NA in the substantia gelatinosa with ejection in the vicinity of the neuron being tested. Ionophoretic application of NA into lamina II resulted in a selective reduction of nociceptive responses in 50% of the cells tested. Ejection of NA in the vicinity of the neuron being recorded from, however, resulted in a much less selective effect and, on this basis, the authors proposed that different receptor populations are activated following NA application at the two sites.

The selective inhibitory action exerted by NA upon dorsal horn neurons has been shown to be mediated through an α_2-adrenoceptor. The specific α_2-agonists clonidine, metraminol and tizanidine all mimick the action of NA, whereas the α_1-agonists phenylephrine and amidephrine (and the ß-agonist isoprenaline) have no effect (Davies and Quinlan, 1985; Fleetwood-Walker et al., 1985).

Furthermore, the depressant action of NA is reversed by prior administration of the α_2-antagonists yohimbine and idazoxan, but not by the α_1-antagonists prazosin or WB4101 and the ß-blocker sotalol (Davies and Quinlan, 1985; Fleetwood-Walker et al., 1985). The α_2 receptor mediation of these effects is consistent with results from behavioural studies, where analgesia produced by i.t. injection of NA occurs via activation of α_2 receptors.

The ionophoresis studies mentioned so far have concentrated on the effects of NA upon cells in the deeper laminae (III-V) of the dorsal horn. However, Todd and Millar (1983), using carbon fibre electrodes, specifically made recordings from units in laminae I and II of the cat dorsal horn, and found that almost half of these cells were excited by NA. They proposed that these superficial cells belong to a different population than those recorded in previous ionophoretic experiments, and suggested that at least some of these neurons may be inhibitory interneurons which block the high threshold inputs to the deeper cells. This idea is consistent with the findings of Headley et al. (1978), who found NA to be more selective at inhibiting nociceptive pathways when ejected into lamina II, than when applied in lamina V close to the unit being recorded from. However, a number of subsequent studies have challenged the results of Todd and Millar (1983). For instance, Howe and Zieglgänsberger (1987) found that only 20% of units in

the superficial dorsal horn of rat are excited by NA. Most units (70%) were inhibited. Similarly, Millar et al. (1993) found, in the same species, that only 14% of cells were excited by clonidine. Furthermore, North and Yoshimura (1984), making intracellular recordings from rat spinal cord slices, found that the majority of lamina II cells (80%) were hyperpolarized by NA. Excitatory effects were very rare (<3%) and were attributable to α-1 receptors. These later studies suggest that the actions of NA in the superficial dorsal horn are similar to those in the deeper laminae, although excitatory effects may be slightly more common. Millar et al. (1993) found that excitation produced by clonidine in laminae I and II neurons of the rat was specific for low threshold units which were spontaneously active. Non-spontaneously active low threshold units were not affected by clonidine, while multireceptive and high threshold units were both inhibited. However, Howe and Zieglgänsberger (1987) have observed excitation in some multireceptive cells in the rat superficial dorsal horn following NA ionophoresis.

The effects of NA on spontaneous activity or excitation generated by glutamate and the excitant amino acid DL-homocysteic acid (DLH) are equivocal. Some groups report that NA (and AD) usually inhibit spontaneous activity and glutamate or DLH-induced firing (Belcher et al., 1978; Carlton et al., 1991; Davies and Quinlan, 1985), whereas other have found it to have no effect on these parameters (Fleetwood-Walker et al.,

1985). Yet others have shown it can amplify spontaneous activity (Millar et al., 1993). Howe and Zieglänsberger (1987) have recorded cells in the rat dorsal horn which responded to NA in each of these ways.

It has been shown that a positive synergism exists between spinal α_2-adrenergic and opioid systems. Co-administration of morphine with dexmedetomidine (Sullivan et al., 1992) or clonidine (Omote et al., 1991) produces a greater inhibition of nociceptive responses than a simple additive interaction. The use of selective μ or δ opioid antagonists has produced conflicting results as to which opioid receptor mediates these effects. Omote et al. (1991) have suggested that the synergism involves the δ opioid receptor, which accords with results obtained in algesic tests (Roerig et al., 1992), whereas Sullivan et al. (1992) have proposed that the μ opioid receptor is involved.

2. Dopamine.

Using a similar approach to their investigations with NA, Fleetwood-Walker et al. (1988) examined the actions of DA ionophoretically applied to multireceptive neurons in the cat and rat spinal dorsal horn. In the cat, all neurons tested were identified as belonging to the spinocervical tract and were located in laminae III-V, while in the rat, spinocervical tract and spinomesencephalic neurons located in laminae I and III-V were tested. DA consistently and selectively inhibited

neuronal responses to noxious cutaneous stimulation, while the responses to innocuous stimuli were unaffected. This selective antinociceptive effect was exerted in rats (32/34 units tested) and cats (15/17 units tested). The actions exerted by DA in this study were reversed in the presence of sulpiride, a highly selective D-2 DA receptor antagonist. Sulpiride alone had no effect on any responses monitored and failed to modify the antinociceptive effect of clonidine. The high degree of antagonism of the action of DA by sulpiride (>80%) suggests that DA acts primarily at D-2 DA receptors. Consistent with this idea, the D-2 receptor agonist RU24213 mimicked the action of DA, but the D-1 agonist SKF38393 had no effect.

(c). **Results from brainstem stimulation studies.**

Although behavioural and ionophoretic studies provide strong evidence that the descending catecholaminergic pathways regulate the transmission of sensory information through the dorsal horn, conclusive proof of their involvement can only be achieved by observing the effects of electrically or chemically stimulating brainstem nuclei known to contain CA-containing cell bodies.

1. Locus coeruleus.

A number of studies, in cat and rat, have demonstrated that electrical stimulation in the region of the locus coeruleus (LC: A6 cell group) can modify the response of spinal dorsal horn neurons to peripheral stimulation

(Hodge et al., 1983; Liu and Zhao, 1992; Mokha et al., 1985; Zhao and Duggan, 1988). The main effect observed from stimulation in the region of the LC is a pronounced, and somewhat selective, inhibition of activity generated by noxious stimuli. For instance, Hodge et al. (1983) found that 23/26 nociceptive specific cells were inhibited by LC stimulation, but only 28/41 units responding only to hair movement were depressed. In addition, in multireceptive neurons the response to noxious stimuli was almost always inhibited (20/22 units), whereas the response to hair movement was usually left intact (inhibited in only 30% of units). In accord with these findings, LC stimulation has been reported to markedly attenuate excitation generated by C fibres, but leave the non-nociceptive responses, from impulses in Aαß fibres, largely unaffected (Liu and Zhao, 1992; Mokha et al., 1985; Zhao and Duggan, 1988).

A highly significant result to emerge from these studies was the insensitivity of inhibition from the LC, in both cat and rat, to reversal by adrenergic blocking agents. Hodge et al. (1983) found that coerulospinal inhibition was unaltered by the depletion of spinal NA, brought about by repeated lumbar i.t. injection of 6-OHDA, or the depletion of whole brain and cord NA, produced by reserpine. Furthermore the α_2 antagonists idazoxan and yohimbine failed to block the LC inhibition, regardless of whether they were administered ionophoretically, i.v. or topically (Liu and Zhao, 1992;

Zhao and Duggan, 1988). At present, the basis for this lack of dependance on NA is unknown. One possible explanation for this effect is that most descending LC cells do not contain NA. However, the overwhelming majority of descending LC cells in the rat are noradrenergic (Westlund et al., 1983), even if many of those in the cat are not (Stevens et al., 1982). A single hypothesis that explains the insensitivity of coerulospinal inhibition to NA depletion and α_2 blockade, is that other transmitters are co-released with NA, and depletion of NA alone is insufficient to reduce a postsynaptic action. Candidate co-transmitters are enkephalin, neuropeptide Y and galanin. Immunocytochemical studies have shown that a proportion of the noradrenergic cell bodies in the rat LC contain neuropeptide Y (Everitt et al., 1984; Holets et al., 1988; Hunt et al., 1981a; Sawchenko et al., 1985), and that some of these cells (about 2%) project to the spinal cord (Holets et al., 1988). Furthermore, the majority of LC profiles are positive for galanin (Holets et al., 1988; Melander et al., 1986), and some of these also (about 3%) project to the spinal cord (Holets et al., 1988). In addition, LC neurons have been shown to contain enkephalin (Charnay et al., 1982). The contribution of these peptides to effects seen in the spinal cord after electrical stimulation of the LC is unknown but, if synergistic with NA, a suppression of the action of NA may be insufficient to reduce the effects of LC stimulation. Significantly, therefore, the candidate

co-transmitters galanin and neuropeptide Y have been reported to depress nociceptive reflexes in rats (Hua et al., 1991; Yanagisawa et al., 1986).

This hypothesis on its own cannot explain the insensitivity of inhibition from the LC to NA depletion brought about by 6-OHDA. This agent destroys the noradrenergic terminals which actively take it up. Thus, release of the putative co-transmitter, as well as NA, will be blocked. It has been suggested that two populations of neurons may project to the spinal cord from the LC (Hodge et al., 1983), one which contains both NA and neuropeptide and another which contains only neuropeptide. 6-OHDA would destroy only the former population of cells, an action which may be insufficient to reduce the effects of LC stimulation due to the existence of the second population of cells. However, since virtually all LC neurons contain NA, cells positive only for neuropeptide Y or galanin are rare (Everitt et al., 1984; Holets et al., 1988). Thus, the possibility that dorsal horn neurons receive two inhibitory inputs from the LC (only one of which is dependant upon NA) is unlikely.

The antinociceptive effect of LC stimulation on dorsal horn neurons has been substantiated in behavioural studies. Electrical stimulation within the region of the LC was shown to increase the threshold to perceive noxious heat as assessed by the hot-plate (Segal and Sandberg, 1977) and tail-flick (Jones and Gebhart, 1986)

tests. Jones and Gebhart (1986) found that inhibition of the tail-flick response was reduced by intrathecal yohimbine, confirming that the LC-induced analgesia was, at least in part, mediated by NA (an observation which does not accord with the results described above). Mapping studies confirmed that pontine sites requiring the lowest intensities of stimulation to produce analgesia were within the LC (Jones and Gebhart, 1986). Furthermore, these authors showed that glutamate, microinjected into the LC, mimicked the electrically-induced analgesia, confirming that the descending inhibition was the result of activating cell bodies rather than fibres of passage. This elevation of nociceptive threshold could be additionally increased by systemic morphine (Segal and Sandberg, 1977).

Sasa et al. (1974) have examined the influence of LC stimulation on activity generated in cat trigeminal nucleus (TN) neurons. Activation of LC neurons decreased the amplitude of TN field potentials, suggesting that fibres originating in the LC inhibit these cells. Pretreatment of the animals with reserpine rendered LC stimulation ineffective at reducing orthodromic spike generation, and demonstrated the dependance of this effect upon NA (Sasa et al., 1974). This finding is fundamentally different to what has been reported for the coerulospinal projection (Hodge et al., 1983; Liu and Zhao, 1992; Zhao and Duggan, 1988). This disparity is surprising since the trigeminal dorsal horn is the first relay station for dental and facial primary afferents,

and is anatomically analogous to the spinal dorsal horn (Yonehara et al., 1990). On this basis, it might have been anticipated that LC projections to the two regions would be equivalent.

2. A7 group

In addition to the LC, electrical stimulation in the region of the A7 cell group, in the cat (Zhao and Duggan, 1988) and primate (Girardot et al., 1987), has been reported to depress sensory transmission. However, in contrast to the effects reported for LC stimulation, activation of descending axons from the SC-PBN (Girardot et al., 1987) and KFN (Zhao and Duggan, 1988) produced a non-selective inhibition of both high and low threshold inputs to dorsal horn neurons. This inhibition from the KFN was resistant to reversal by yohimbine (Zhao and Duggan, 1988), in the same manner as LC-induced inhibition in the same study.

This attenuation of nociceptive responses by the A7 group has also been reported in a behavioural test. Yoemans et al. (1992) have found that focal electrical stimulation in the rgion of the A7 group, in the rat, increased the latency of foot withdrawal to noxious radiant heat. This effect was blocked by i.t. administration of phentolamine or yohimbine, suggesting that it was mediated by NA.

3. A5 group.

Noradrenergic neurons located in the A5 cell group also appear to regulate nociceptive responses. Thus, electrical and chemical stimulation of neurons in this area has been shown to inhibit the tail-flick reflex in rats (Burnett and Gebhart, 1991; Millar and Proudfit, 1990). This antinociceptive action was blocked by i.t injection of α-antagonists.

4. A1 group.

Stimulation in the region of the lateral reticular nucleus, in rats, selectively inhibits C-fibre inputs to dorsal horn neurons (Liu and Zhao, 1992). Topically, or intra-venously, applied yohimbine markedly reduces this effect.

5. A11 group.

Selective inhibition of nociceptive pathways has also been reported following activation of descending DA fibres from the A11 cell group (Fleetwood-Walker et al., 1988). Since sulpiride, but not α_2 blockers, antagonised this effect, it was concluded that this was a specific D-2 DA receptor mediated action.

6. Other groups.

Electrical stimulation within the nucleus raphe magnus (NRM) and the nucleus reticularis paragigantocellularis (NRPG), two medullary nuclei implicated in antinociception, has been shown to increase the efflux of

NA from the spinal cord (Hammond et al., 1985). This increased efflux is due to the activation of NA nuclei by the NRM and NRPG. Furthermore, microinjection of morphine into the NRPG increases the concentration of normetanephrine, a metabolite of NA, in the spinal cord (Kuraishi et al., 1978). These results establish that excitation within noradrenergic nuclei in the brain can cause a release of NA within the spinal cord.

(d). **Mechanisms of action.**

Although the actions of CA's upon dorsal horn neurons have been described in detail, very little is known about the possible mechanisms through which these actions are mediated. However, evidence has been presented that both presynaptic and postsynaptic mechanisms are involved.

1. Evidence favouring a postsynaptic mechanism of action.

North and Yoshimura (1984) discovered that NA has a direct hyperpolarizing action upon rat substantia gelatinosa neurons in vitro, an effect which is blocked by α-2 receptor antagonism. These authors suggested that this postsynaptic hyperpolarization may account for the inhibitory action of NA upon dorsal horn neurons activated by sensory stimulation. Engberg and Ryall also observed this hyperpolarization in their pioneering study of 1966. They found that NA restored the spike amplitude of action potentials reduced by excessive depolarization.

Consistent with the idea of a postsynaptic mechanism

of action are the results from several studies investigating the effects of NA, or clonidine, upon dorsal horn neurons excited by glutamate or D-homocysteic acid (DLH) (Belcher et al., 1978; Carlton et al., 1991; Engberg and Ryall, 1966; Millar et al., 1993). The basis of these studies is that if NA acts exclusively at presynaptic sites, to inhibit transmitter release, it would be expected to have no effect upon amino acid-induced excitation. On the other hand, if NA acts partially at postsynaptic sites, to depress the neurons excited by glutamate or DLH, it should decrease the effects of these compounds. A number of groups have found that NA and clonidine inhibit cells excited by these amino acids (Belcher et al., 1978; Carlton et al., 1991; Engberg and Ryall, 1966; Millar et al., 1993). However, Fleetwood-Walker et al. (1985, 1988) found NA and DA to be inactive in this test.

Hylden and Wilcox (1983) have modified this approach into a behavioural assay. It is now generally accepted that substance-P (SP) is one of the neurotransmitters released by nociceptive primary afferent fibres (Besson and Chaouch, 1987), and it has been demonstrated that i.t. injection of SP elicits behavioural responses similar to those induced by peripheral irritation (Hylden and Wilcox, 1983). NA applied i.t. after SP, reduced the biting and scratching episodes associated with injection of SP alone. This favours the idea that NA acts postsynaptically to inhibit the dorsal horn cells acted upon by SP, rather than preventing its presynaptic

release from primary afferent terminals.

2. Evidence favouring a presynaptic mechanism of action.

Several lines of evidence have suggested that NA may also act presynaptically upon the terminals of primary afferent neurons.

Single fibre excitability testing studies in cats have shown that ionophoresis of NA into the dorsal horn (Carstens et al., 1987; Jeftinija et al., 1981) and electrical stimulation of brainstem areas known to contain NA (Carstens and Zimmermann, 1981) can raise the antidromic threshold for activation of C and A fibres. Such an increase in electrical threshold has been suggested to represent presynaptic inhibition, but, if so, it is not like that produced by GABA (i.e. primary afferent depolarization). Jeftinija et al. (1981) found that the rise in threshold was dose dependant: ionophoretic application of NA with 50nA current increased the threshold value to 190% of control, whereas NA at smaller current strengths produced lower increases in threshold (137% with 25nA and 126% with 15nA). This increase in electrical threshold was resistant to reversal by phentolamine or yohimbine (Jeftinija et al., 1981). Curtis et al. (1983) found that NA also increased thresholds in muscle Ia afferents. However, since this effect could be reversed by ouabain, the authors suggested that it was not mediated by adrenoceptors located presynaptically on Ia afferent

terminals. They proposed that the rise in threshold was an artifact of changes in extracellular ionic composition due to active neural uptake of NA via a ouabain sensitive pump. Wohlberg et al. (1985) have reported that NA hyperpolarizes frog primary afferent terminals.

A limitation of excitability testing is its failure to prove unequivocally that transmitter release is actually reduced. However, Kuraishi et al. (1985a) have reported that perfusion of the rabbit dorsal horn with a solution containing NA completely inhibits the noxious pinch-induced release of substance P. Similarly, perfusion with dexmedetomidine, an α_2 agonist, has been shown to significantly decrease the capsaicin-evoked release of substance P and calcitonin gene-related peptide from the rat dorsal horn (Takano et al., 1993). In addition, NA and the α_2 agonist clonidine reduce the K^+-evoked overflow of glutamate from rat spinal cord synaptosomes (Kamisaki et al., 1993). These inhibitory effects were reversed by yohimbine (Kamisaki et al., 1993; Kuraishi et al., 1985a; Takano et al., 1993).

In vitro studies, using cultured chick sensory neurons, have shown that NA decreases the duration of somatic action potentials evoked by K^+, an effect which can be attributed to a lowering of the inward calcium current (ICa) (Dunlap and Fischbach, 1981). If NA was to affect the ICa in the nerve terminals in the same way, then it may reduce transmitter release. In contrast to these findings, Gallagher et al. (1980) have reported that DA depolarizes the somata of feline sensory neurons.

If DA was to affect the axon terminals in this way, then it may reduce transmitter release by producing primary afferent depolarization (PAD) (Burke and Rudomin, 1977). However, it is unknown whether these somatic events can be extrapolated to the axonal arbor.

4. NEUROPEPTIDE Y.

(a). **Isolation and relationship to other peptides.**

Neuropeptide Y (NPY) is a 36 amino acid peptide which was first isolated and sequenced from extracts of pig brain by Tatemoto and his collegues in 1982 (Tatemoto, 1982a; Tatemoto et al., 1982). Its name is derived from the high content of tyrosine (Y) residues within its primary structure.

A comparison of the primary structure of NPY with peptide YY (PYY) and avian and bovine pancreatic polypeptides (APP and BPP) indicates that a high degree of sequence homology exists between them. BPP (Lin and Chance, 1974), APP (Kimmel et al., 1975) and PYY (Tatemoto, 1982b) share 17, 20 and 25 amino acids respectively in identical positions to NPY, and many of the remaining residues seem to be compensating, i.e. they occur between structurally similar amino acids. It was therefore proposed that NPY, PYY and PP are members of a family of peptides, which may have evolved from one common ancestor (Tatemoto, 1982a). PP has been shown by immunohisto-chemistry to be located in endocrine cells of

Langerhans' islets (Larsson et al., 1976), and PYY is found within endocrine cells of the gastrointestinal mucosa, particularly in the colon (Lundberg et al., 1982).

Subsequent analyses have found that antisera raised against APP or BPP are also capable of producing immunostaining in the central nervous system (Hökfelt et al., 1981; Hunt et al., 1981a,b; Jacobowitz et al., 1982; Lundberg et al., 1980; Olschowka et al., 1981). However, when these antisera were employed in radioimmunoassay, little, if any, APP or BPP could be measured in rat (Allen et al., 1983; DiMaggio et al., 1985; Lundberg et al., 1984) or human (Adrian et al., 1983) brain. These conflicting results could be explained by the presence of a peptide in the brain, distinct from PP, which cross-reacts with PP antisera, and therefore immunostains, but competes poorly with synthetic PP in radioimmunoassay.

When antisera were subsequently raised against NPY, they were found to label the same structures as previously recognised with APP or BPP antisera (Adrian et al., 1983; Allen et al., 1983; Chronwall et al., 1985; deQuidt and Emson, 1986), and evidence is now substantial that NPY is the endogenous PP-like peptide in the mammalian central nervous system (Adrian et al., 1983; Allen et al., 1983; DiMaggio et al., 1985; Lundberg et al., 1984). Earlier reports on the distribution of APP- and BPP-like like peptides were the result of antibody cross-reactivity due to the large sequence homology with NPY, and should therefore be considered studies of NPY-

immunoreactivity.

Although PP is absent from nervous tissue, it is present in large amounts in a special type of cell within the pancreatic islets (Larsson et al., 1976; Lundberg et al., 1984), where, on release, it exerts both paracrine and endocrine actions (Lin and Chance, 1974). Similarly, PYY is found within gastrointestinal endocrine cells (Lundberg et al., 1982, 1984; Tatemoto, 1982b), where it too serves, at least partly, as a local hormone. In contrast, NPY is not present in pancreatic or intestinal endocrine cells (Lundberg et al., 1984; Sundler et al., 1983) and it has been suggested (Tatemoto et al., 1982) that NPY is found only in nerve cells and that PP and PYY are found only in endocrine glands. Subsequent studies have essentially confirmed this suggestion to be correct, with the exception that a few neurons in the brainstem, at least in the rat, contain PYY (DiMaggio et al., 1985; Lundberg et al., 1984).

(b). **Neuropeptide Y in the spinal cord.**

For the purposes of this review, papers describing the distribution of APP- or BPP-immunoreactivity in the spinal cord are considered studies of NPY.

1. Distribution of NPY-immunoreactive (NPY-IR) axons.

NPY-IR axons have been shown to innervate the spinal cord of a variety of mammals, including rat (Chronwall et al., 1985; deQuidt and Emson, 1986; Gibson et al., 1984;

Hökfelt et al., 1981; Hunt et al., 1981a,b; Sasek and Elde, 1985; Wakisaka et al., 1991), cat (Gibson et al., 1984; Krukoff, 1987), rabbit (Blessing et al., 1987), guinea-pig (Gibson et al., 1984), horse (Merighi et al., 1990), pig (Merighi et al., 1990), marmoset (Gibson et al., 1984) and human (Allen et al., 1984). In each species, these structures were found throughout the grey matter at all segmental levels of the cord, but the heaviest innervation of NPY-IR axons was found in the thoracic intermediolateral cell column. They were more abundant in the lumbosacral region than in cervical segments and this cranio-caudal gradient was most prominant with respect to the ventral horn.

(a). *Dorsal horn.*

In the dorsal horn of most species (cat excepted), extremely heavy concentrations of NPY-IR axons and varicosities were found within the superficial layers (laminae I and II) (Allen et al., 1984; Blessing et al., 1987; Gibson et al., 1984; Hökfelt et al., 1981; Hunt et al., 1981b; Merighi et al., 1990; Sasek and Elde, 1985), although in the rat there is some disagreement concerning the precise distribution of NPY-IR in this region. Hunt et al. (1981b) and Sasek and Elde (1985) reported that the highest concentrations were in lamina I and the outer (dorsal) part of lamina II, whereas Hökfelt et al. (1981) found that NPY-IR fibres were less dense in lamina I than in lamina II. In cats, lamina I has been reported to be devoid of NPY-IR terminals (Krukoff, 1987), with the

highest numbers of NPY axons being reported in laminae II and III (Gibson et al., 1984; Krukoff, 1987). In all species, the deeper layers of the dorsal horn (IV-VI) were found to contain only low to moderate numbers of NPY-positive fibres (deQuidt and Emson, 1986; Gibson et al., 1984; Hökfelt et al., 1981; Hunt et al., 1981b; Krukoff, 1987; Sasek and Elde, 1985). This distribution of NPY terminals in the dorsal horn is similar to that of NPY binding sites mapped by autoradiography (Kar and Quirion, 1992).

(b). *Ventral horn.*

The density of NPY-IR fibres in the ventral horn varied considerably depending upon the segmental level. In cervico-thoracic segments only single varicose fibres were seen, whereas in the lumbosacral region large numbers of NPY-IR axons were observed (Allen et al., 1984; deQuidt and Emson, 1986; Gibson et al., 1988; Hökfelt et al., 1981; Hunt et al., 1981a; Krukoff, 1987; Sasek and Elde, 1985). These axons were most numerous in the vicinity of the large motoneurons (lamina IX), with Onuf's nucleus (which is thought to innervate perineal striated muscle) receiving a particularly heavy innervation (Gibson et al., 1984).

(c). *Autonomic nuclei.*

A dense network of NPY-IR fibres was seen in close association with the cells of the thoracic and sacral

sympathetic and parasympathetic preganglionic nuclei (Blessing et al., 1987; deQuidt and Emson, 1986; Hökfelt et al., 1981; Krukoff, 1987; Sasek and Elde, 1985). From each of these nuclei a prominant band of fibres ran in a medial direction, joining in the midline dorsal to the central canal.

(d). *Area adjacent to the central canal.*

A dense innervation of NPY-IR fibres surrounds the central canal in lamina X. The intensity of this innervation was heaviest dorsal to the central canal (Gibson et al., 1984; Hökfelt et al., 1981; Merighi et al., 1990; Sasek and Elde, 1985), and in thoracic and sacral segments it formed a plexus which merged with the band of fibres that extended between the central canal and the autonomic nuclei (see above).

2. Distribution of NPY-IR perikarya.

The injection of colchicine into the dorsal horn (Hunt et al., 1981a,b) or intrathecally (deQuidt and Emson, 1986; Krukoff, 1987; Sasek and Elde, 1985), to raise the level of NPY in cell bodies, has revealed large numbers of NPY-synthesizing perikarya within the spinal cord of rat and cat.

In the rat (deQuidt and Emson, 1986; Hunt et al., 1981a,b; Sasek and Elde, 1985), IR perikarya were detected in large numbers in laminae I-III throughout the cord. Only occasional NPY-positive cells were found in the remainder of the grey matter, although the sacral

dorsal grey commissure did contain a prominant population of NPY neurons. There is some disagreement concerning the precise segmental distribution of NPY-IR cells in the rat. Some authors (deQuidt and Emson, 1986; Sasek and Elde, 1985) have suggested that the cervical enlargement (40 cells per 50μm section) contains more NPY-IR neurons than the lumbosacral region (20 cells per 50μm section), although others have reported that staining in the two regions was similar (Hunt et al., 1981b). Recently, Rowan et al. (1993), using a very sensitive immunostaining procedure, have labelled large numbers of NPY-IR neurons (20 per 50μm section) in laminae I-III of the lumbar cord without the use of colchicine. The authors found that all of these neurons also contained GABA.

The distribution of NPY-positive perikarya in the cat is different to that observed in rats (Krukoff, 1987). IR cell bodies were only present below segment T2, where they were concentrated in laminae VII, with additional cells in laminae VI, VIII and IX. The greatest number of IR cell bodies (up to 18 in any given 50μm section) were found in the sacral cord. No cell bodies were observed in laminae I-V at any segmental level.

In the rabbit (Blessing et al., 1987) and pig (Merighi et al., 1990), a few NPY-IR cell bodies have been observed in laminae I-III of the dorsal horn without the use of colchicine.

3. Other possible origins of spinal cord NPY terminals.

(a). *Primary afferent neurons.*

Immunocytochemical studies in the rat, guinea-pig, horse, pig and marmoset (Gibson et al., 1984; Hökfelt et al., 1981; Merighi et al., 1990; Wakisaka et al., 1991), have produced no evidence for the presence of NPY in dorsal root ganglion cells, despite the fact that other peptides such as substance P, calcitonin gene-related peptide and galanin were readily detected (Hökfelt et al., 1991; Merighi et al., 1990). These findings, though must be interpreted cautiously, since the levels of NPY within primary afferent neurons may simply be too low to be detected immunocytochemically. However, it is generally thought that NPY is not present in these structures, at least in the species noted above (Weihe, 1990). In accordance, following a number of surgical and pharmacological procedures, which destroyed primary afferent fibres, no changes were seen in the density and distribution of NPY-IR axons in the dorsal horn, nor was there any change in the amounts of NPY that could be extracted from it (Gibson et al., 1984; Hökfelt et al., 1981). In contrast, substance P levels on the ipsilateral side were markedly depleted. These observations point to an entirely intrinsic origin for spinal NPY-IR axons in these species, which may be derived from supraspinal cell bodies and/or spinal neurons.

In the cat, however, a small proportion of spinal NPY-

containing fibres may be derived from sensory neurons. Lindh et al. (1989) have shown that a few dorsal root ganglion cells in the cat contain NPY, and NPY-containing fibres have been observed in Lissauers tract and in the dorsal roots in this species (Gibson et al., 1984).

(b). *The brain.*

An obvious potential source of spinal NPY terminals are neurons whose cell bodies are located in supraspinal sites, and radioimmunoassay & immunocytochemical studies have demonstrated a widespread distribution of NPY-immunoreactivity throughout the brain of many species including rat (Allen et al., 1983; Chronwall et al., 1985; deQuidt and Emson, 1986; Everitt et al., 1984; Hendry et al., 1984; Holets et al., 1988; Sawchenko et al., 1985), cat (Coveñas et al., 1990), rabbit (Blessing et al., 1986) and primate (Hendry et al., 1984; Smith et al., 1985) including man (Adrian et al., 1983; Halliday et al., 1988). Indeed, NPY was present in higher concentrations than any other brain peptide hitherto discovered. Furthermore, NPY-IR perikarya were located in several areas of the brain which were known to project to the spinal cord, including the brainstem noradrenergic nuclei (Blessing et al., 1986; Everitt et al., 1984; Halliday et al., 1988; Hökfelt et al., 1983a,b; Holets et al., 1988; Hunt et al., 1981a; Lundberg et al., 1980; Sawchenko et al., 1985). However, results from retro-

grade tracing studies suggest that only NPY neurons located in the locus coeruleus (Holets et al., 1988) and the C1 catecholamine cell group of the medulla (Blessing et al., 1987) descend to the spinal cord. Furthermore, it was shown that projections from these regions to the dorsal and ventral horns are only minor. Thus, of the many hundreds of NPY-IR perikarya located in the locus coeruleus, only 2% were found to project to spinal cord (Holets et al., 1988). Furthermore, NPY neurons in the C1 group were shown to project mainly, if not entirely, to the sympathetic lateral cell column (Blessing et al., 1987; Ross et al., 1984). In accordance with these findings, no obvious changes in the distribution or density of NPY-IR fibres could be seen in the dorsal or ventral horns below a complete or hemi cord transection (Hökfelt et al., 1981; Hunt et al., 1981b), whereas many of the NPY-IR fibres in the thoracic sympathetic nucleus disappeared (Hökfelt et al., 1981).

(c). **Functional studies.**

The presence of high numbers of NPY axons and NPY receptors in the superficial dorsal horn suggests that NPY may regulate nociceptive sensory processes. It is significant, therefore, that NPY has been shown to produce analgesia in conscious rats following intrathecal administration (Hua et al., 1991). Although numerous studies exist describing the effects of NPY in the periphery and in the brain (reviewed by Gray and Morley, 1986), physiological studies of the actions of NPY on

spinal neuronal systems are sparse. However, there are several lines of evidence which suggest that the analgesic action of NPY may be brought about, at least in part, by a presynaptic inhibitory action upon the terminals of nociceptive primary afferent axons within the dorsal horn. For instance, it has been demonstrated that rat sensory neurons in culture possess unusually high concentrations of specific binding sites for NPY, and that activation of such receptors inhibits the K^+ stimulated release of substance P from these cells (Bleakman et al., 1988; Walker et al., 1988). Similarly, microinjection of NPY into the dorsal horn of cats inhibits the C-fibre evoked release of substance P (Duggan et al., 1991). Furthermore, the number of NPY binding sites in the superficial dorsal horn is dramatically reduced when the primary afferent input to this region is removed by dorsal rhizotomy or neonatal capsaicin treatment (Kar and Quirion, 1992). At present, there are no publications reporting the effects of NPY upon dorsal horn neurons.

Schwartz et al. (1989), using radiolabelled NPY and C-terminal fragments of the NPY molecule, have characterized two types of NPY receptor. The first of these, the Y1 receptor present on PC-12 cells, required the whole NPY structure for binding. The second receptor, the Y2 type, is present in rat hippocampus and recognises not only the whole NPY molecule, but also C-terminal fragments (e.g. NPY_{13-36}). These two types of

receptor are somewhat similar to the two types of receptor found on dorsal root ganglion cell membranes (Walker et al., 1988). One receptor had a high affinity for NPY (Kd=0.08nM), whereas the other site had a much lower affinity (Kd=6.5nM). Fuxe et al. (1990) have suggested different functional roles for high and low affinity NPY receptors. Low affinity receptors (possibly Y1) are proposed as being localized to the synaptic regions where high amounts of the non-degraded peptide are encountered. High affinity receptors (possibly Y2) are proposed as occuring remotely from the sites of synaptic release and are dependant upon diffusion of released NPY through the extracellular fluid. They term this process volume transmission. A proportion of released NPY would be degraded by peptidases and hence Y2 receptors are likely candidates for mediating the remote effects of released NPY because they are activated by C-terminal fragments of NPY. The spinal NPY receptor mediating thermal antinociception (Hua et al., 1991) appears to be of the Y2 type.

(4). OBJECTIVES OF THE STUDY.

The effects produced by catecholamines upon sensory transmission are very well documented (sections 3a-c), but the circuitry underlying these effects is very poorly understood (section 3d), although both presynaptic and postsynaptic mechanisms have been suggested.

The present studies were prompted by the need to

improve our understanding of the mechanisms through which descending catecholaminergic neurons, terminating within the spinal dorsal horn, regulate sensory transmission. In the first of these studies, the ultrastructural organization of spinal catecholamine-containing nerve fibres was examined using an antiserum to tyrosine hydroxylase (TH). TH is the enzyme which converts tyrosine to dihydroxyphenylalanine (DOPA), and is the first enzyme in the catecholamine biosynthetic pathway (Fig.1). Therefore, it is an endogenous enzyme of dopaminergic, noradrenergic and adrenergic neurons. Results obtained in these experiments were supplemented with data from a second immunocytochemical study employing an antiserum to dopamine-ß-hydroxylase. The justification for this second study was that only noradrenaline-containing axons (and scant adrenaline-containing axons) would be labelled by this antiserum. Dopaminergic fibres would not be stained. Furthermore, owing to the recent discovery of neurons in the brain that contain TH, but not catecholamine (Vincent and Hope, 1990), it cannot be assumed that all TH-positive structures are catecholaminergic.

In these analyses, the postsynaptic targets of catecholamine-containing nerve terminals were not identified, and a major goal of subsequent studies was to identify the dorsal horn neurons receiving synaptic input from these descending pathways. Consequently, projection neurons of the postsynaptic dorsal column pathway and the

spinocervical tract were retrogradely labelled by means of a horseradish peroxidase implantation technique (Enevoldson and Gordon, 1989a,b), and catecholamine-containing axons were immunocytochemically stained as before. In addition, the possibility of a catecholaminergic innervation to the lateral cervical nucleus was also examined.

It has been suggested, on the basis of the extensive co-localization of NPY with catecholamines in spinally projecting brainstem nuclei (Everitt et al., 1984), that many fibres in the dorsal horn may contain both neurotransmitters (Duggan et al., 1991). Therefore, an examination was also made of axons containing neuropeptide Y, to assess whether co-storage between catecholamines and this peptide is likely in the spinal cord.

Materials and methods.

Adult cats (2.3-3.9kg), of either sex, were used in all of these experiments.

1. PERFUSION FIXATION OF CATS.

The animals were deeply anaesthetized with sodium pentobarbitone (40mg/kg) administered intraperitoneally. Then, once the corneal and limb withdrawal reflexes had disappeared, the trachea was located and canulated with a Y-shaped connector. The animal was then transfered to a perfusion apparatus, where the thorax was rapidly opened and the ribs retracted to reveal the heart. At this stage the animal was connected, via the tracheal canula, to a respiratory pump. The pericardium was gently removed and an incision was made into the left ventricle, through which a steel canula was inserted, guided into the aorta and clamped into place. This steel canula was connected to the perfusion flasks.

The animal was then perfused with warm saline (37°C) containing 100U/ml heparin and 0.1% sodium nitrite. The solution was delivered at a pressure of 120mmHg and, once the blood had cleared, 1000ml of warm fixative (37°C) was introduced at the same pressure. The fixative consisted of 4% paraformaldehyde, 0.1% glutaraldehyde and 15% saturated picric acid in 0.1M phosphate buffer, pH 7.4. A further 2000ml of fixative was delivered at 100mmHg and 4°C.

Berod et al. (1981) have shown that increasing the pH of the fixative enhanced tyrosine hydroxylase immunohistofluorescence in the caudate nucleus. Therefore, in one experiment, the cat was perfused with a solution of 4% paraformaldehyde in 0.1M borate buffer at pH 9.0.

Following perfusion, lumbosacral segments (L6-S1) of the spinal cord were removed and kept in the same fixative (with the omission of glutaraldehyde) for a further 4-6hrs. The blocks were washed and stored overnight in phosphate buffered saline (PBS). Transverse and sagittal sections (40μm) of lumbosacral spinal cord were cut with a Vibratome (Oxford Laboratories or Horwell) and rinsed with several changes of PBS to ensure complete removal of excess fixative.

2. IMMUNOCYTOCHEMICAL STAINING.

(a). **Specificity of primary antisera used.**

The primary antisera used in the these studies specifically recognise tyrosine hydroxylase, dopamine-ß-hydroxylase or neuropeptide Y.

The tyrosine hydroxylase (TH) antiserum was raised in rabbits from TH extracted from bovine adrenal glands, and its characteristics have been described in detail by van den Pol et al. (1984). Briefly, its specificity for TH was determined by precipitation of enzyme extracts from a crude cell homogenate (Western blot): only a single band of 62,000 Daltons was recognised by this antiserum.

Immunohistochemical specificity was confirmed by the abolition of immunostaining following pre-incubation with TH, but not any other antigens. Therefore, only regions of the brain known to contain catecholamines were found to react with this antiserum (Freund et al.,1984; van den Pol et al., 1984).

The antiserum to dopamine-ß-hydroxylase (DBH) was produced by Eugene Tech International, Inc. (New Jersey, USA), and was purchased direct from the manufacturer or through Affiniti Research Products Ltd., UK. The antiserum was raised in rabbits from DBH purified from bovine adrenals, and its specificity was confirmed, by the maunufacturers, using Western blotting and immunocytochemistry. The specificity of this antiserum has also been reported by Chang (1989).

The neuropeptide Y (NPY) antiserum was manufactured by Cambridge Research Biochemicals, in rabbits, from porcine NPY, and the characteristics and specificity of this antiserum have been described by Polak and Bloom (1984). Briefly, negligible cross-reactivity was observed with other members of the pancreatic polypeptide family and immunostaining was only found in tissue known to contain NPY.

(b). **Immunostaining procedure.**

Vibratome sections were immersed in 10% normal donkey serum (NDS) with 0.01% sodium azide in PBS for 30min to reduce non-specific (background) staining by secondary antisera. This was followed by overnight incubation in

the primary antiserum at 4°C. The antisera were diluted 1:1000 (anti-TH and anti-DBH) or 1:2000 (anti-NPY) in PBS containing 1%NDS and 0.01% azide. For anti-DBH, this solution was also supplemented with 0.3% Triton X-100. Although ultrastructural preservation is compromised by the addition of this agent to the incubation medium (since it disrupts lipid membranes), its inclusion is essential for successful DBH-immunocyto-chemical staining (Chang, 1989; Lewis & Morrison, 1989). As Triton X-100 also increases antibody penetration, a number of sections to be treated with anti-TH and anti-NPY were pretreated with 0.3% Triton X-100 for 1hr prior to incubation in the primary antiserum.

TH, DBH and NPY were visualized in the test sections by using the avidin-biotin-peroxidase complex (ABC) technique (Hsu et al., 1981). This involved incubation for 45min in donkey anti-rabbit biotinylated immuno-globulin G (Amersham) diluted 1:100 at room temperature, followed by 15min in streptavidin peroxidase complex (Amersham) diluted 1:300 at room temperature. The tissue was washed between steps in PBS and dilutions were made in PBS containing 1% NDS. The presence of peroxidase was visualized by reaction with hydrogen peroxide in the presence of 3,3-diaminobenzidine (DAB). The sections were placed in a 0.1% solution of DAB dissolved in 0.5% Tris buffer (pH 7.5) and gently agitated for 20min. Hydrogen peroxide was added to achieve a final concentration of 0.01% and the tissue was incubated for a further 6-10min

at room temperature.

(c). **Controls.**

The primary antiserum was omitted from the incubation medium in control experiments.

In addition, tissue from the striatum and the locus coeruleus was removed, and sections were incubated with anti-TH or anti-DBH and processed identically to the lumbosacral test sections. The striatum possesses a dense dopaminergic network (Lindvall and Björklund, 1983), and may thus be used to assess the specificity of the antisera. It would be anticipated that the anti-TH would produce a strong immunoreaction in striatal sections, while anti-DBH would produce a negative result. The locus coeruleus contains large numbers of noradrenergic perikarya (Lackner, 1980; Westlund et al., 1983), and thus both TH and DBH antisera would be expected to label cell bodies in this region.

(3). ELECTRON MICROSCOPY.

Following the DAB reaction, the immunostained sections were postfixed in 1% osmium tetroxide for 1hr and dehydrated through a series of ethanol solutions for the times shown (50% for 5min; 70% for 40min; 95% for 15min; 100% for 15min twice). "En bloc" staining was performed with a 1% solution of uranyl acetate in 70% ethanol. After dehydration, the sections were cleared in propylene oxide (two 10min rinses), and placed in fresh Durcurpan

in a dessicator for 2-3 hrs. They were then flat-embedded in Durcurpan between cellulose acetate foils and polymerized at 60°C for 48hr. When polymerization was complete the sections were examined with the light microscope for the presence of immunostained axons, and regions of interest were photographed or drawn with the aid of a drawing tube.

Sections to be examined with the electron microscope were subsequently attached to Durcurpan blocks and thin sections (pale gold interference fringe; 70-90nm) were cut using a diamond knife on a Reichert-Jung ultramicrotome. Ribbons of serial thin sections were collected on single-slot copper grids coated with Formvar and were stained with Reynold's lead citrate for 2-3min. Immunostained fibres were examined through these series, with the electron microscope (Phillips EM400), to determine the synaptic arrangements that they formed.

The criteria for identifying a region as a synaptic junction were as follows (Gray, 1959): (i) the presynaptic axon terminal was separated from the apposed postsynaptic structure by an obvious synaptic cleft (20-30nm), (ii) the vesicles in the presynaptic structure were clustered in the region of the synaptic cleft and could occasionally be seen attached to the presynaptic membrane, (iii) the pre- and postsynaptic membranes displayed densities on their cytoplasmic faces, which were either of equal density (symmetric, or Gray type II, synaptic junction) or were greater on the postsynaptic

membrane (asymmetric, or Gray type I, synaptic junction).

Maximum diameters of boutons, diameters of dendrites in contact with them and lengths of synaptic appositions were measured directly from electron micrographs using a Reichert videoplan system.

(4). RETROGRADE LABELLING TECHNIQUES.

These experiments were designed to identify some of the dorsal horn neurons that are postsynaptic to catecholamine-containing axons. Consequently, projection neurons of the postsynaptic dorsal column pathway and the spinocervical tract were retrogradely labelled by means of a horseradish peroxidase (HRP) implantation technique. This procedure was performed by Dr. D.J. Maxwell, with myself in attendance.

(a). **The postsynaptic dorsal column (PSDC) pathway.**

Postsynaptic dorsal column neurons were retrogradely labelled according to the method described by Enevoldson and Gordon (1989a). Cats were deeply anaesthetized with pentobarbitone (40mg/kg, i.p.) and a dorsal laminectomy was performed at T10-T12 (3 animals) to expose the spinal cord. Strict aseptic conditions were maintained throughout this procedure and normal body temperature was maintained by an electric blanket controlled by a rectal probe. An incision was made in the dura mater and excess cerebrospinal fluid was removed by suction. The spinal cord was kept dry by using tissue paper wicks. Small

bilateral lesions were made in the midline region of the dorsal columns with the aid of a razor blade knife, and one or two small agar-HRP pellets were placed into the lesion with dry watchmaker's forceps. When the pellet was in position, the lesion was plugged with small pieces of Sterispon (Allen and Hanbury Ltd, UK) and a drop of quicksetting cyanoacrylate glue (Loctite, UK) was used to seal the implant site. At the conclusion of surgery, the spinal cord was covered with Sterispon and the incision was sutured in layers. The animals were given intramuscular injections of penicillin (100,000 units) and allowed to recover from the anaesthetic.

In one cat, a unilateral HRP implant was made in the dorsal columns of the rostral cervical cord (C2-C3). A lesion was then made in the ipsilateral dorsolateral funiculus (DLF) one segment caudal to the implant site to ensure that neurons projecting to the lateral cervical nucleus with axons in the DLF were not transporting HRP.

The animals were allowed to survive for 36-72 hours (T10-T12 implants) or 96 hours (C2-C3 implant). They were then transcardially perfused as described above, and the implant site was recovered for histological analysis, together with lumbosacral segments L6-S1. The segments containing the implant site (T10-T12 or C2-C3) and the segment containing the control cervical DLF lesion (C4) were embedded in 0.3% agar and sectioned transversely with a freezing microtome (Leitz) at 90μm. Serial frozen sections of the implant sites were treated with DAB and

hydrogen peroxide for visualization of HRP around the implant site. They were then mounted, counterstained with neutral red and examined with the light microscope. Sections from the DLF lesion were treated similarly, except that they were not processed for peroxidase. Drawings were constructed from a number of serial sections through the implant sites and the control DLF lesion to illustrate their maximum extent.

Transverse and sagittal sections of lumbosacral spinal cord were cut with a Vibratome, and retrogradely labelled neurons within these sections were also visualized with DAB as the chromogen. The sections were then wet-mounted in buffer on glass slides and scanned with the aid of a light microscope. Sections containing labelled neurons were processed for TH- and DBH-immunocytochemistry as previously described. They were then osmicated, dehydrated and embedded in Durcurpan between cellulose acetate foils. When polymerization was complete the sections were examined with the light microscope for possible contacts between retrogradely labelled neurons and immunostained axons. A site was designated a region of contact when no space could be discerned between the varicosity and the cell at a magnification of X1000. The contacts were then photographed or drawn with the aid of a drawing tube and the sections were processed for correlated electron microscopy.

(b). **The spinocervical tract (SCT).**

SCT neurons, in 2 cats, were retrogradely labelled

according to the method described by Enevoldson and Gordon (1989b). The surgical procedure was similar to that described for the labelling of PSDC neurons, and only notable differences will be highlighted here. In these experiments, a unilateral lesion was made in the dorsal columns at C4-C5 to ensure that neurons projecting through the dorsal columns to the dorsal column nuclei did not transport HRP. A second small lesion was then made in the ipsilateral DLF one segment rostrally and a pellet of HRP was inserted into the lesion. After a 96hr survival period the animals were anaesthetized for a second time and perfused with fixative. Retrogradely transported HRP was visualized according to the histochemical method described above, although in some experiments sections were incubated in 5% cobalt chloride prior to the DAB reaction (Adams, 1977), in order to intensify the HRP reaction product within labelled cells. The immunostaining procedure was then carried out as previously described for PSDC neurons.

Results.

(1). ANALYSIS OF AXONS IMMUNOSTAINED FOR TYROSINE HYDROXYLASE (TH) OR DOPAMINE-ß-HYDROXYLASE (DBH).

(a). **Light microscopic observations.**

The density and distribution of axons and varicosities immunostained for TH and DBH were comparable and will be described together. Nerve fibres immunoreactive for TH and DBH were found in all laminae of the spinal dorsal horn, but within each lamina differences were noted with respect to the density and trajectory of the axons and varicosities.

In transverse sections of the dorsal horn, immunoreactive fibres were most abundant in laminae I-II and the medial part of lamina IV (Fig.3B). Within laminae I and II, TH- and DBH-immunoreactivity was observed primarily as isolated punctate profiles or short axons with few *en passent* varicosities (Figs.6D;7E;8B;13B), although long strands of catecholaminergic axons were sometimes seen travelling in the dorsoventral plane (Figs.6I;8B,C;14B). In laminae III & IV, immunoreactive axons were primarily short and varicose, and ran dorsoventrally (Fig.7B), mediolaterally (Figs.7D,F) or followed highly curved paths (Figs.7A,C).

Analysis of the distribution of TH- and DBH-immunolabelled axons in the sagittal plane revealed large numbers of rostrocaudally oriented axons within laminae I and II (Figs.3A;4A,B;6A,B,F,G). These fibres were highly

varicose (Fig.4A;6A,B,F,G), and could often be followed for several millimeters (Fig.4A). The rostrocaudal orientation was most prominant in lamina I. In lamina II and the outer portion of lamina III the predominant orientation of the labelled axons was still rostrocaudal, but many axons travelled at an angle to the dorsal border of the dorsal horn (Figs.4A;5;6C,H), and some axons followed a highly curved path (Fig.4B). Large numbers of immunoreactive fibres were also found in the inner portion of laminae III and in lamina IV, but these axons were predominantly oriented dorsoventrally (Fig.3A).

TH- and DBH-immunopositive fibres may be classified into at least two distinctive populations on the basis of the size of their varicosities. The most common type of axon had both large and small varicosities intermingled along its length (Figs.4A;6A-H,J;7A,B,D-F), while the second, less common type was characterized by small varicosities, which were only slightly larger than their intervaricose axons (Figs.6I;7C,H;18F). Throughout the dorsal horn, large numbers of immunoreactive varicosities were found which were in contact with neuronal somata (Figs.4A,B;5;6A;7E-H).

Additional TH- and DBH-positive axons formed a dense plexus around the central canal. However, the remainder of the grey matter, including the ventral horn, contained very few immunoreactive axons (Fig.3A,B). No TH- or DBH-immunoreactive perikarya were observed in these studies.

The high pH fixation regime recommended by Berod et

al. (1981) produced inconsistent results. In a few sections a very dense immunoreaction was observed (Fig.5), which was comparable to the staining seen in sections that had been treated with Triton X-100. However, in most sections only very weak immunolabeling was observed. In addition, this fixation produced very poor ultrastructural preservation.

(b). **Ultrastructural observations.**

(1). TH-immunoreactive axons.

Correlated ultrastructural analysis confirmed that these varicosities were synaptic boutons. In all, 107 boutons exhibiting TH-immunoreactivity were examined through serial sections with the electron microscope. Terminals were predominantly round or oval in shape and varied in size from 0.40μm to 1.88μm in diameter. A frequency distribution histogram of bouton diameters is shown in Fig.12A. The majority of profiles (84%) fell within the range 0.6-1.2μm (0.95μm mean diameter). TH-containing boutons characteristically formed single synapses on dendrites (88%) or neuronal cell bodies (6%). They were not observed to form axo-axonic synapses, although they were occasionally found closely apposed to other vesicle-containing structures. A few boutons (6%), when followed through serial sections, did not form synaptic junctions. TH-positive boutons were packed with small, irregularly shaped, agranular vesicles (Figs.8D;-9D,E;10A-D,F;11C), together with several dense-core

vesicles (Fig.11C). The boutons usually also contained at least one mitochondrion. The immunoreactive material within labelled boutons was most obvious along the outer membranes of small synaptic vesicles and mitochondria. Dense core vesicles did not appear to be positive for TH.

Synaptic junctions formed by these axon terminals were of the symmetric type (Gray type II; Gray, 1959), and were small (mean±s.d.= 0.35±0.12μm) compared to the diameter of the bouton. Axo-dendritic synapses were found throughout laminae I-VI of the dorsal horn. TH boutons synapsed most commonly with medium-sized (0.5-2.0μm diameter) dendrites (Figs.8D;10C-D;12B). Large calibre dendritic shafts (>2.0μm diameter) also received synapses from TH-immunoreactive axonal endings (Figs.9D;10E,F;-12B), as did small (<0.5μm diameter) dendrites or spines (Figs.9E;10A,B;12B). Symmetric axo-somatic synapses were encountered in laminae II-IV. No examples were observed in laminae I or V-VI, but fewer immunolabelled boutons were sampled in these regions. A small immunoreactive bouton in lamina IV in association with a large neuronal perikaryon is shown in Fig.11B. At higher magnification (Fig.11C) the synapse is revealed.

(2). DBH-immunoreactive axons.

The ultrastructural organization of axons positive for DBH was similar to that described above for terminals containing TH. In total, 36 DBH-immunoreactive boutons were examined through serial sections with the electron

microscope and, although tissue preservation was not optimal due to treatment with Triton X-100, sufficient detail was retained to examine synaptic organization.

Noradrenaline-containing boutons usually formed single synapses on dendrites (75%) or somata (3%), although two boutons formed synapses with more than one postsynaptic target. DBH terminals formed synapses most commonly with medium-sized (0.5-2.0μm diameter) dendrites (Figs.13E; 14E,F), but contacts upon small (<0.5μm diameter) dendrites and dendritic spines (Fig.13G) were not uncommon. Immunolabelled boutons were not observed to form axo-axonic arrangements. A large number of DBH-positive boutons (22%), when followed through serial sections, did not form synaptic junctions, but this was due to tissue disruption associated with Triton X-100 pretreatment. Synaptic junctions formed by these axon terminals were usually of the symmetric type (Gray type II), with a slight accumulation of electron dense material on the presynaptic and postsynaptic sides of the junction. One synapse (Fig.14F) appeared to be Gray type I (asymmetric), although in serial section it looked symmetric (Fig.2E). Non-synaptic puncta adhaerentia were also seen (Fig.13C). Boutons contained small, irregularly-shaped agranular vesicles (Fig.13D,F), which were markedly depleted in number due to treatment with Triton X-100, together with several dense-core vesicles (Fig.13D,E). DBH-immunoreactivity was associated with both types of vesicle.

(2). CONTROLS.

Both TH (Fig.15A,B) and DBH (Fig.15F,G) antisera labelled neuronal cell bodies in sections from the locus coeruleus (A6 noradrenergic cell group). Sections from the striatum that had been treated with the TH antiserum displayed a prominant network of fibres (Fig.15C,D), which was absent from the sections incubated with anti-DBH (Fig.15H,I). Spinal cord sections that had been incubated in a medium from which the TH (Fig.15E) or DBH (Fig.15J) antiserum had been omitted displayed no immunolabelling.

(3). CATECHOLAMINERGIC INNERVATION OF POSTSYNAPTIC DORSAL COLUMN NEURONS.

(a). **Implant sites.**

The implant sites from four cats are shown in Fig.16. Drawings were constructed from a number of serial sections through the lesion and thus represent the maximum extent of the lesion. Although there was a little damage to the dorsal regions of the grey matter in some sections, the transections were otherwise confined to the dorsal columns. The lesions never extended as far as the central canal and never involved the lateral funiculi. These reconstructions confirmed that the thoracic implants (T10-T12) were bilateral (Figs.16A-C) and that the cervical implant (C2-C3) was confined to the

right hand side of the cord (Fig.16D). The reaction product of HRP from the pellet was most intense in the region of the implant, but some reaction product was usually observed surrounding it in the grey matter and the DLF. The control lesion, caudal to the cervical implant, was accurately made in the DLF at C4 (Fig.16E).

(b). **Retrograde labelling.**

The distributions of retrogradely labelled neurons were comparable to those reported in previous studies (Bennett et al., 1983; Enevolson & Gordon, 1989a; Giesler et al., 1984; Rustioni, 1976; Rustioni & Kaufman, 1977), but differences may exist between labelling produced by implants at the two regions of the spinal cord. Bilateral HRP implants in the thoracic cord produced large numbers of labelled neurons within laminae III, IV and medial V. A much smaller second concentration of labelled cells was found in the medial portions of laminae VI and VII and an occasional cell was observed in lamina I. These cells were found in roughly equal numbers on both sides of the cord. The unilateral cervical implant produced a much more restricted distribution of labelling, although a much smaller population of neurons was sampled. With the exception of a single neuron in lamina VII, all retrograde labelling was confined to laminae III-V.

The retrogradely transported HRP granules were present in the perikarya and proximal dendrites of labelled neurons (Figs.18;19). Labelled primary and secondary

dendrites were observed routinely but tertiary dendrites were rare. Axons were sometimes labelled, but their collaterals were not apparent. It was noted that cells labelled from the cervical implant (Fig.19) usually contained less HRP reaction product than those labelled from the more caudal implant (Fig.18).

(c). **Light microscopic observations of immunoreactive varicosities and postsynaptic dorsal column neurons.**

Large numbers of axons immunoreactive for TH and DBH were observed throughout the lumbosacral spinal dorsal horn but, in accordance with earlier studies, they were particularly abundant within the superficial layers (laminae I and II) and medial lamina IV. It was noted that the density of immunostaining produced with the DBH antiserum was greater than that observed with the anti-TH, even when comparing TH-labelled material treated with Triton X-100.

All retrogradely labelled neurons examined for immunoreactive contacts were within laminae III-V. Sixty-seven out of one- hundred-and-seven cells (62.5%), labelled following the thoracic implants, possessed DBH-immunoreactive terminals closely apposed to their perikarya and/or proximal dendrites (mean number of contacts per cell=4.4, range=1-16). An example is shown in Fig.18E,F. Twenty-three out of thirty-nine cells (59.0%), labelled from the cervical implant received contacts from DBH-immunostained axons (mean number of

contacts per cell=4.9, range=1-22). Examples are shown in Fig.19. One-hundred-and-ninety-seven cells labelled from the thoracic implants, were examined for contacts from TH-immunoreactive axons. Analysis of tissue that had been treated with Triton X-100 revealed that 34% of neurons had terminals apposed to their somata and/or proximal dendrites (mean number of contacts per cell=3.2, range=1-13). Examples are shown in Fig.18A-D (see also tissue prepared for combined light and electron microscopy; Figs.20;21. The apparent lower innervation from axons containing TH is a consequence of the weaker immunoreaction observed with this antiserum.

Fibres immunoreactive for TH and DBH formed both terminal and *en passant* axonal swellings, and individual collaterals gave rise to both single (Figs.18A) and multiple (Figs.18;19) contacts. Frequently, axons could be seen to follow closely the curvature of dendrites or somata (Figs.18C-F;19). Many retrogradely labelled neurons were innervated by several immunostained axons (Figs.18;19A). In accordance with earlier findings, at least two different morphological types of axon can be distinguished by the dimensions of the varicosities which occur along the axonal strands. Both types of axon appear to innervate PSDC cells. The more common type of axon had both large and small varicosities intermingled along its length (Figs.18A-D;19), while the second, less common type was characterized by small varicosities which were only slightly larger than their intervaricose axons (Figs.18E,F).

The laminar location of 44 labelled cells, observed in transverse sections, receiving contacts from TH- or DBH-immunostained contacts is shown in Fig.17. Approximately 70% of the cells are located in the medial half of laminae III-V, where most of the CA-containing fibres are found.

(d). **Electron microscopic observations of immunoreactive varicosities and postsynaptic dorsal column neurons.**

Ultrastructural examination of retrogradely labelled neurons demonstrated that they contained HRP reaction product which was sequestered within membrane-bound granules (Figs.20;21). Such electron-dense bodies are typical of retrogradely transported HRP (La Vail and La Vail, 1974). In addition to membrane-bound HRP some neurons contained diffuse reaction product which was low in intensity and usually associated with membranous structures.

Varicosities observed with the light microscope were revealed by electron microscopic analysis to be synaptic boutons. The appearance of these boutons was comparable with those reported in earlier analyses. They contained large numbers of small, round or oval agranular vesicles together with several dense core vesicles. Using correlated light and electron microscopy, 12 CA-containing boutons were confirmed to form synaptic specializations with the dendrites (Fig.21) and somata (Fig.20) of labelled cells. These synapses could be

followed for up to 9 serial sections.

On 8 occasions, axonal swellings which formed apparent contacts at the light microscopic level were found not to make synaptic associations with retrogradely labelled structures when viewed with the electron microscope. Seven of these structures synapsed upon an adjacent, unlabelled structure. The remaining bouton did not form an identifiable synaptic junction and lay closely apposed to the soma of a retrogradely labelled cell.

(4). CATECHOLAMINERGIC INNERVATION OF SPINOCERVICAL TRACT NEURONS.

The cervical HRP implant sites are shown in Fig. 22A & C. Drawings were constructed from a number of serial sections through the lesion and thus represent the maximum extent of each implant site. Although there was a little damage to the grey matter in each case, the transections were otherwise confined to the DLF.

The distributions of retrogradely labelled neurons (n=316) in the lumbosacral (L6-S1) spinal cord were comparable to those reported in previous studies (Brown et al., 1980a, 1977b; Enevoldson and Gordon, 1989b). Most HRP-positive cells were found in laminae III (31%), IV (50%) and V (13%), but occasional cells were labelled in laminae I, II, VI and VII (6%). Two hundred and ninety eight HRP-labelled neurons, within laminae III-V, were examined for contacts from TH- or DBH-immunoreactive axons and 13% of these cells had immunopositive terminals

directly apposed to their somata and/or proximal dendrites. The results obtained with each antiserum were pooled since both produced a similar frequency of contact. Most immunolabelled contacts were single (Fig.23B-D), but occasionally a varicose axon was seen to follow the curvature of a dendrite or soma. The maximum number of contacts observed on a single labelled neuron was 7. The omission of Triton X-100 from the incubation medium reduced TH-immunostaining to the extent that no examples of contacts between TH-positive axons and HRP-labelled neurons were found in this tissue.

(5). ANALYSIS OF AXONS IMMUNOSTAINED FOR TYROSINE HYDROXYLASE IN THE LATERAL CERVICAL NUCLEUS.

(a). **Light microscopic observations.**

TH-immunoreactive (TH-IR) fibres were seen throughout the LCN (Fig.24). Long strands of TH-positive axons were occasionally observed (Fig.25A) but most profiles appeared as isolated punctate varicosities (Figs.25B;26) or short axons with few *en passant* swellings. Throughout the LCN, TH-IR varicosities were seen in contact with neuronal somata (Fig.26), including large cells (30-50μm diameter) in the lateral two-thirds of the nucleus. However, the majority of labelled terminals were not associated with cell bodies but were found dispersed within the neuropil.

Th-IR neurons were not observed in the LCN (or in the

cervical spinal cord) in these experiments.

(b). **Ultrastructural observations.**

In total, 54 boutons exhibiting TH-immunoreactivity were examined through serial sections with the electron microscope. Terminals were predominantly round or oval in shape and varied in size from 0.49-1.25μm in diameter (mean diameter=0.77μm). A frequency distribution histogram of bouton diameters is shown in Fig.29A. The boutons were packed with small round or oval agranular vesicles (Figs.26B;27B-D,F;28) and often contained one or more large dense-core vesicles (Figs.26B;27B;28C,F).

The majority of TH-IR boutons (87%) formed synaptic junctions, which were of the symmetric type (Gray type II) and were small (mean±s.d.=0.27±0.07μm) in relation to the diameter of the bouton. CA-containing boutons commonly formed synapses with medium-sized (0.5-2.0μm diameter) dendrites (85% of synapses; Figs.27B-D;28C,D 29B). Large dendrites (>2.0μm diameter) also received synapses from TH-IR axonal endings (3% of synapses; Figs. 28E,F;29B), as did small (<0.5μm diameter) dendrites and spine-like structures (6% of synapses; Figs.27D;28A,B 29B). Synapses were also found upon small (<20μm diameter; Fig.26) and large (>20μm diameter; Fig.27E,F) LCN neuronal cell bodies (6% of synapses).

TH-positive boutons were not observed to form axo-axonic synaptic junctions, although they were often found closely apposed to other unlabelled axon terminals.

(6). ANALYSIS OF NEUROPEPTIDE Y-IMMUNOREACTIVE PROFILES IN THE DORSAL HORN.

(a). **Light microscopic observations.**

The distribution of NPY-immunoreactive (NPY-IR) fibres throughout the spinal dorsal horn (laminae I-VI) is shown in Fig.30. NPY-IR axons and varicosities were found in all six laminae but within each lamina differences were noted with respect to their density and appearance.

A dense plexus of NPY-containing axon terminals was observed within the superficial laminae (Fig.30A,B,G,H). Moderate numbers of NPY-IR axons were found in laminae III and IV (Fig.30C,D,J) but only occasional, scattered fibres were observed in laminae V and VI (Fig.30E,F).

Within transverse sections of laminae I-III the NPY terminals were present predominantly as single, punctate structures (Fig.30B) and varicose axons were only occasionally observed. Axonal strands within laminae I-III were more common in sagittal sections, where they were usually oriented rostrocaudally (Fig.30G,H). The varicosities dispersed along the length of such a strand were usually small and discrete (Fig.30G). Long beaded NPY-IR fibres were more common in the deeper layers of the dorsal horn (Fig.30D,F,J), particularly in lamina IV, where they often collected into prominant bundles of fibres (Fig.30C). These bundles were particularly evident in transverse sections and they sometimes extended into laminae III and V. Throughout the dorsal

horn, NPY-IR terminals were found in contact with neuronal cell bodies (Fig.30E,G,I).

The remainder of the spinal grey matter, including the ventral horn, was rather sparsely innervated by NPY-containing axons, with the exception of the region adjacent to the central canal (lamina X) which contained a moderate innervation.

NPY-IR cell bodies and dendrites were not observed in the cat spinal cord in these experiments. Control sections, which had been incubated in a medium from which the NPY antiserum had been omitted, displayed no immunolabelling.

(b). **Ultrastructural observations.**

In total, 208 profiles exhibiting NPY-immunoreactivity were examined through serial sections with the electron microscope (Table 2). The overwhelming majority of these structures (n=194) was confirmed to be axon terminals and most of these (95%) formed synaptic junctions. NPY axon terminals were mostly round or oval in shape and varied from 0.4-2.1μm in diameter. However, some terminals were highly flattened and were only slightly wider than there intervaricose axons (Fig.31D,E; see also 30G). These long, thin axons were particularly common in lamina II. Boutons immunostained for NPY were packed with large numbers of small, round or oval vesicles and often contained one or more large dense-core vesicles (Figs. 31D,E;32B;35B;36D,E,F). Immunoreactivity was homogenously scatteres throughout the cytoplasm and was also

associated with the dense-core vesicles. The cores of the small, clear vesicles were not immunoreactive. A frequency distribution histogram of bouton diameters is shown in Fig.39A. The majority of profiles (90%) fell within the range 0.5-1.2μm diameter (mean diameter= 0.75μm).

Many more labelled axon terminals were seen in the superficial dorsal horn when compared with deeper laminae (III-VI), as would be anticipated from the light microscopic observations described above for Triton X-100 treated material. Within laminae I and II, 141 NPY-containing nerve terminals were examined through serial sections and 136 (97%) formed synaptic junctions. The organization of these structures was complex, with 40% of terminals forming more than one synapse. Most labelled terminals were presynaptic to dendrites (70% of synapses) (Fig.31), but some were presynaptic to other axon terminals (27% of synapses) (Figs.32-34) and a few contacted neuronal cell bodies (3% of synapses) (Fig.35).

Axo-dendritic synaptic junctions formed by NPY-IR boutons were symmetric (Fig.31) and occured most commonly with medium-sized (0.5-2.0μm diameter) dendrites (Fig.31B;39B). Large dendrites (2.0μm diameter) also received synapses from NPY-IR axons (Fig.31C;39B), as did small (<0.5 μm diameter) dendrites and spines (Fig.31A; 39B). In transverse sections, postsynaptic dendrites were usually found in cross-section (Fig.31A-C), whereas in sagittal sections they were often oriented rostro-

caudally (Fig.31E-G). Approximately 25% of NPY-IR terminals which formed an axo-dendritic synapse also synapsed with unlabelled axon terminals, and about one-half of these arrangements were confirmed to be synaptic triads (Fig.36).

Analysis of NPY axo-axonic associations revealed that NPY-containing terminals were presynaptic to the unlabelled terminal (Figs.32-34). These unlabelled terminals were sometimes the central boutons of synaptic glomeruli (Figs. 32C.D;34C). As already mentioned above, many NPY axo-axonic synapses were components of triads or complex synaptic arrays. However, 50% of the NPY boutons which formed axo-axonic arrangements synapsed only upon a single axon terminal (Figs.32A,B,D;34A,B). NPY axo-axonic synaptic junctions of both symmetric (Gray type II) (Figs.32,34C,D) and asymmetric (Gray type I) (Fig.34A,B) were observed. No NPY boutons resenbling central boutons of glomeruli were found.

Four NPY-IR terminals in lamina II formed symmetric synaptic junctions upon cell bodies. Two of these boutons were large and formed multiple active zones with neuronal perikarya. In the example shown (Fig.35C,D), the axo-somatic junction was examined through over 30 serial sections.

NPY-IR terminals were much less numerous in the deeper dorsal horn (III-VI) than in the superficial laminae, but 55 immunostained profiles were examined through serial sections. NPY-positive terminals formed axo-dendritic (Fig.37A-D), axo-axonic (Fig.37E-H) and axo-somatic

synapses with roughly the same frequency as observed within laminae I and II. The complexity of the organization of NPY boutons in laminae III-VI, as assessed by their tendency to form multiple synapses, was less than seen in lamina II but similar to that found in lamina I.

Fourteen vesicle-containing NPY-positive profiles examined with the electron microscope were difficult to classify, but they may have been vesicle-containing dendrites. These structures were found within laminae I (n=2), II (n=10) and IV (n=2) and were postsynaptic to a number of unlabelled axon terminals, including a central glomerular terminal in lamina II (Fig.38C). In addition, they were usually presynaptic to one or more dendrites (Fig.38A). Although these structures contained small, round vesicles and large dense-core vesicles, they were few in number.

Figure 3.

A. A composite drawing illustrating the distribution of TH-immunoreactive axons throughout four 40μm sagittal sections of cat lumbosacral (L7-S1) spinal cord. Note the dense innervation in laminae I-IV compared to the rest of the grey matter. In lamina I and the outer region of lamina II the fibres exhibit a prominant rostrocaudal orientation. In laminae III-IV this orientation changes, and most axons are organized dorsoventrally.

B. A composite drawing illustrating the distribution of TH-immunoreactive axons throughout five 40μm transverse sections of cat lumbosacral (L7-S1) spinal cord. Note the particularly high density of fibres in laminae I, II and IV and around the central canal in lamina X.

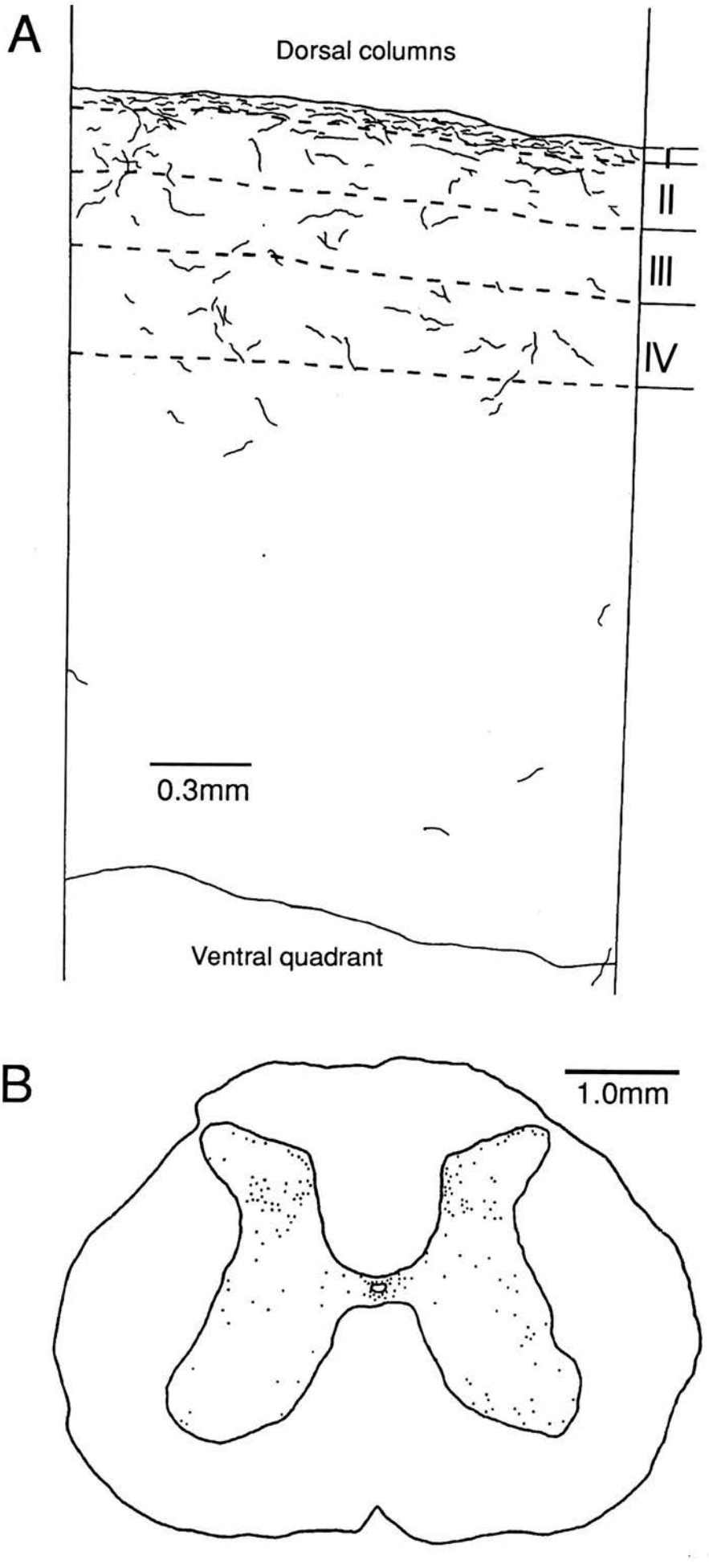

A
Dorsal columns
II
III
IV
0.3mm
Ventral quadrant
B
1.0mm

Figure 4.

A pair of drawings, constructed from sagittal sections treated with Triton X-100, illustrating the morphology and patterns of distribution of TH-immunoreactive axons within lamina II and the outer (dorsal) region of lamina III. **A.** TH-immunoreactive axons travel for considerable distances in a rostrocaudal direction without branching. Most fibres are characterized by regularly spaced varicosities. **B.** Typical features of immunolabelled axons at the lamina II-III border. Many fibres run rostrocaudally while others travel at an angle to the laminar boundary. Further axons follow a highly curved path. Throughout this region, immunolabelled axons are found in contact with neuronal cell bodies (shaded structures).

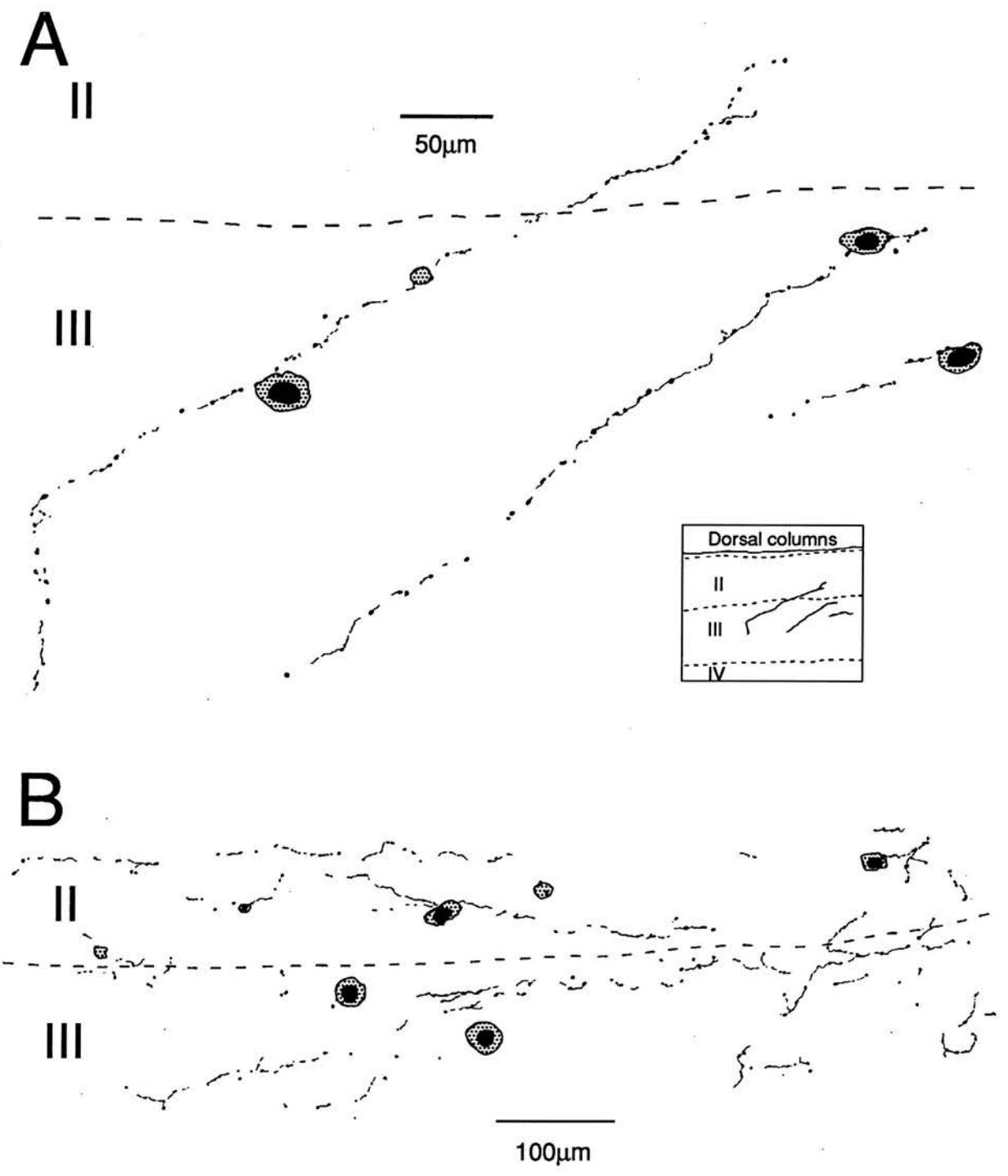

A
II
50μm
III
Dorsal columns
II
III
IV
B
II
III
100μm

Figure 5.

A drawing of TH-immunoreactive axons within a single 40μm sagittal section of spinal cord, which was taken from a cat perfused with high pH fixative (borate buffer, pH 9). In this particular section, the distribution and density of immunoreactivity is comparable to that shown in Fig.4 for material treated with Triton X-100. However, in other sections the high pH fixation regime recommended by Berod et al. (1981) produced only weak immunostaining.

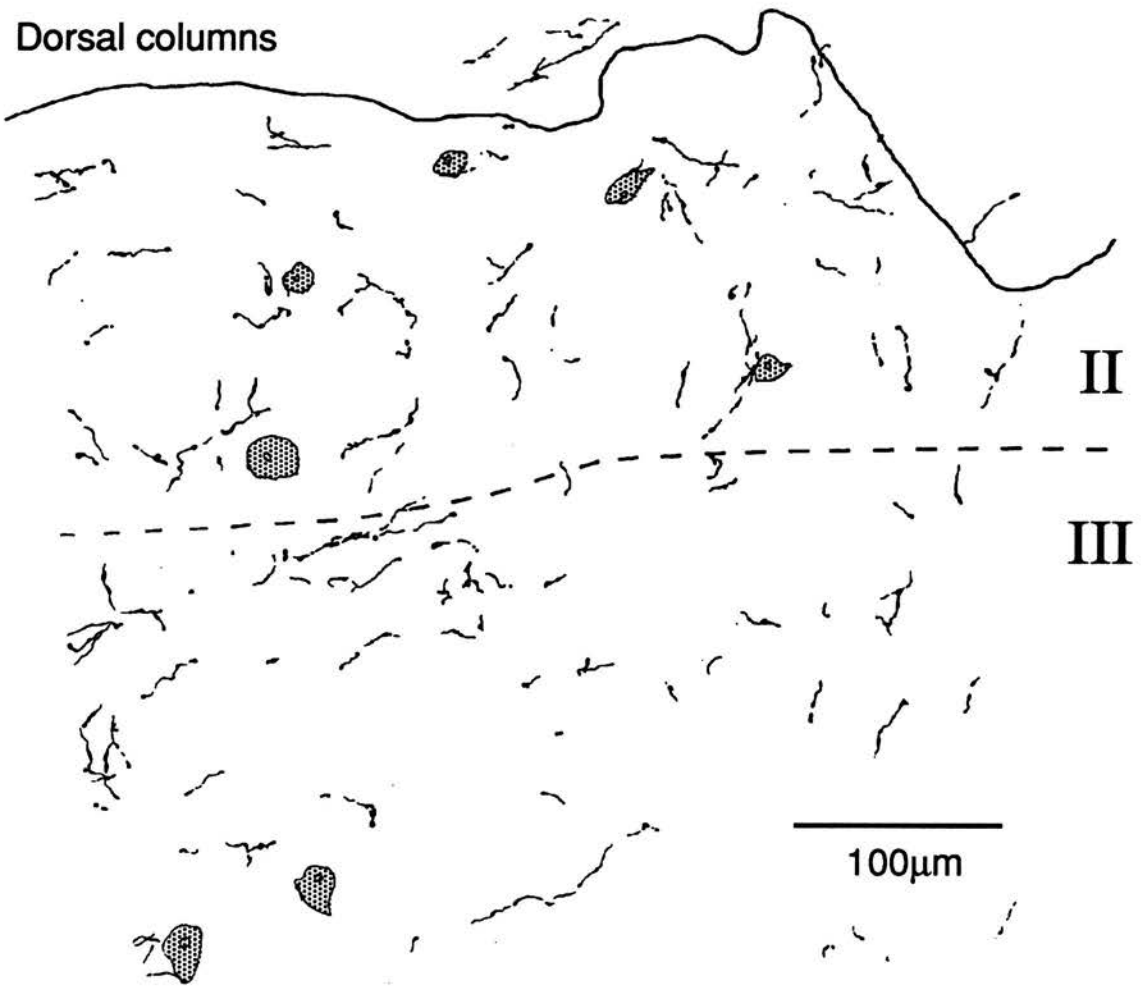

Dorsal columns
II
III
100µm

Figure 6.

Light micrographs illustrating the morphology & patterns of distribution of TH-immunoreactive (A-E) and DBH-immunoreactive (F-J) axons in laminae I and II of the spinal dorsal horn. Many fibres in these layers run rostrocaudally (A,B,F,G) while others travel at an angle to the laminar boundary (C,H). Further axons course dorsoventrally (D,I)) and mediolaterally (E,J).

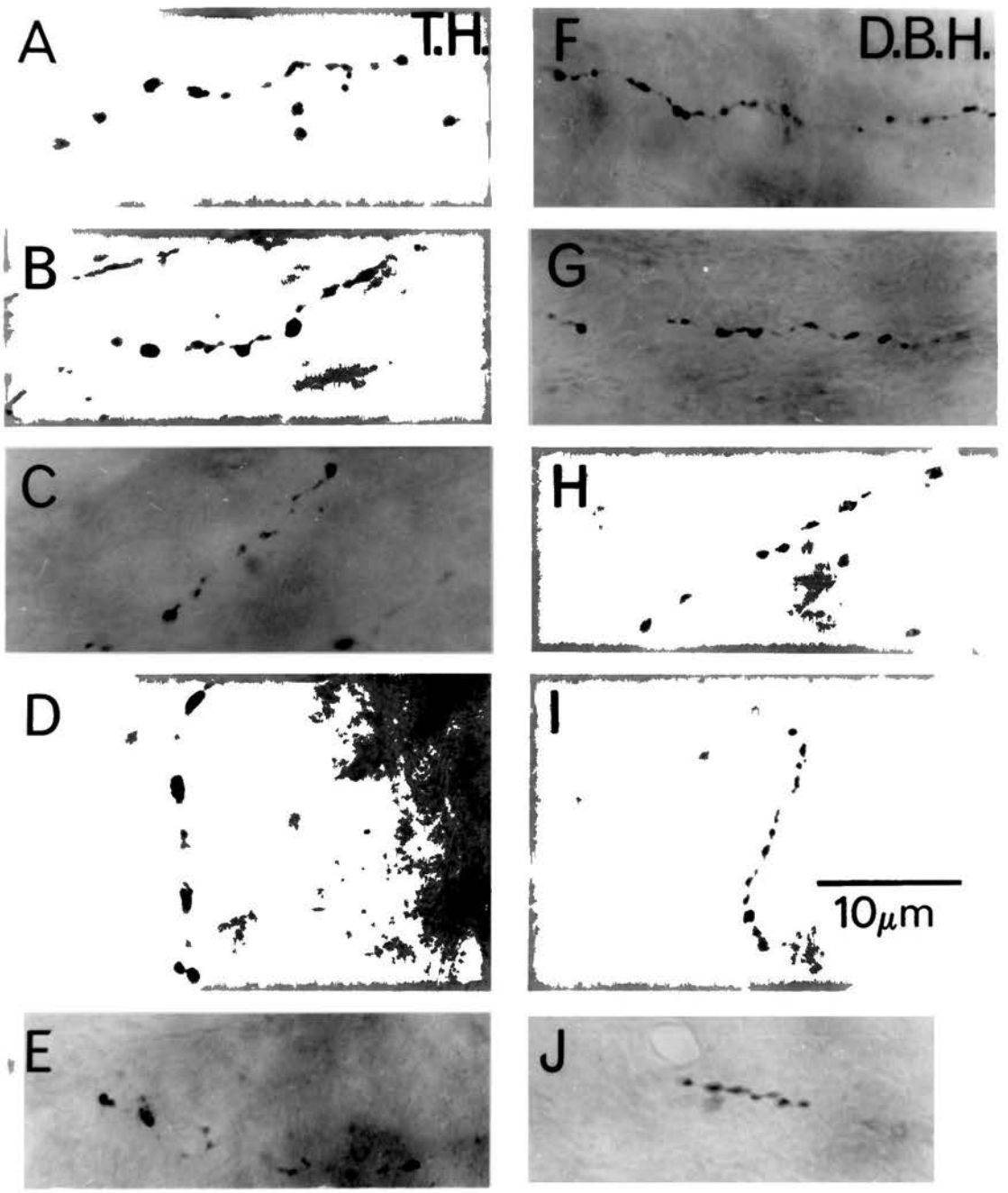

A
T.H.
F
D.B.H.
B
G
C
H
D
I
10μm
E
J

Figure 7.

A-D. Light micrographs illustrating the morphology and distribution of axons immunoreactive for TH (**A,B**) and DBH (**C,D**) in laminae III and IV of the spinal dorsal horn. Many axons run dorsoventrally (**B** and left axon in **D**) while others travel mediolaterally (right axon in **D**). Further axons follow a highly curved path (**A,C**).

E-H. Light micrographs showing TH-Immunoreactive (**E,F**) and DBH-immunoreactive (**G,H**) varicosities (arrows) in laminae II (**E**), III (**F**), IV (**G**) and V (**H**) contacting neuronal cell bodies (*). Scale bar= 10μm (A-D,F-H); 16μm (E).

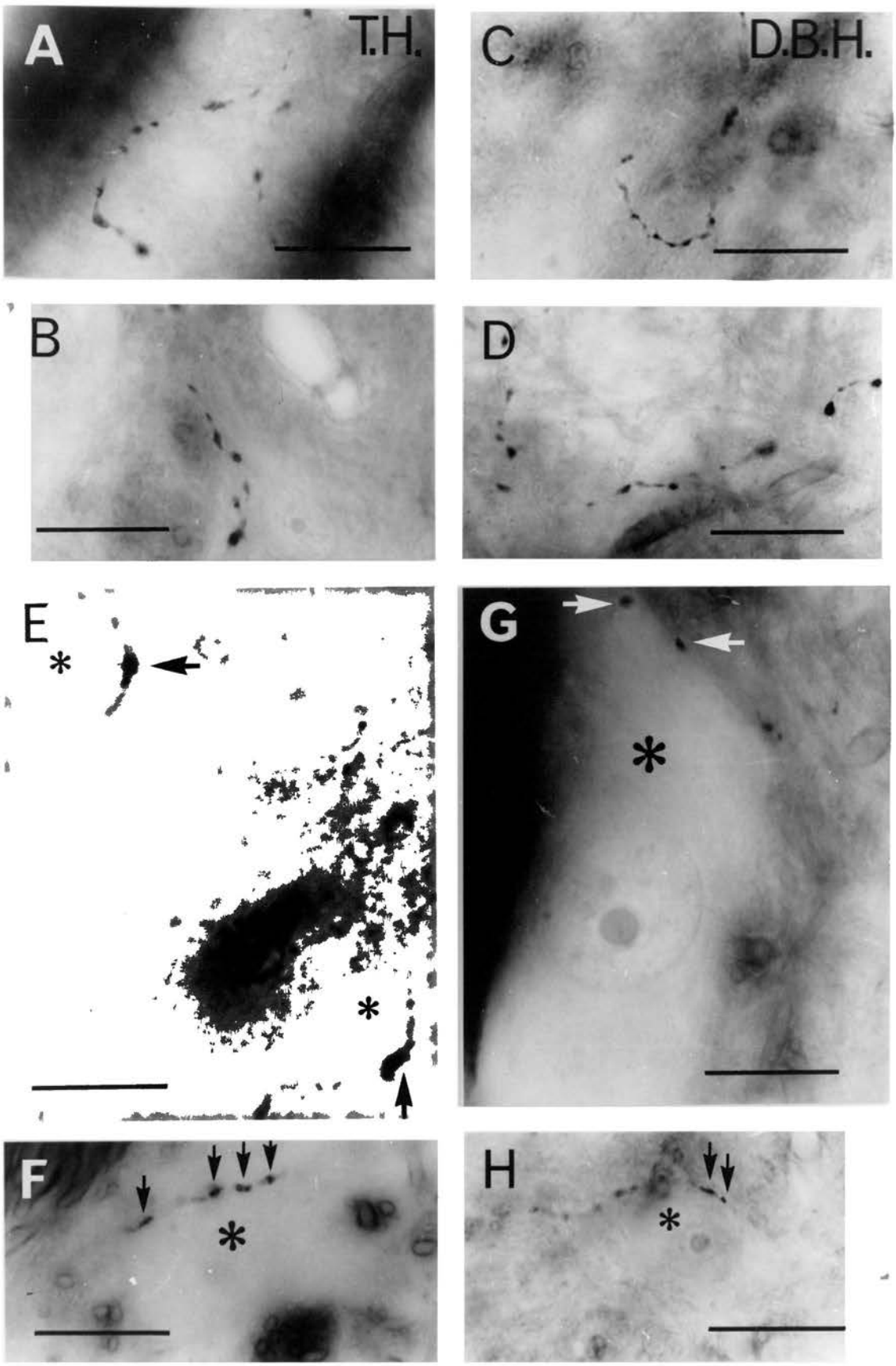

A
T.H.
C
D.B.H.
B
D
E
G
F
H

Figure 8.

A. An outline of the dorsal horn of the L7-S1 region of the spinal cord in transverse section. Higher magnification of the area enclosed by the box is shown in **B.** The distribution of TH-immunoreactive axons within laminae I and II of this region is illustrated for a single 40µm section. In this particular section the density of immunolabelling is similar for both superficial laminae. Surrounded by a small box is an immunostained fibre travelling in the dorsoventral plane. The highly varicose appearance of this axon is shown at higher magnification in **C.** The varicosity marked by an asterisk is shown in an electron micrograph (**D**). This bouton forms a symmetric (Gray type II) synaptic junction (between the arrowheads) with a medium-sized dendrite (D).

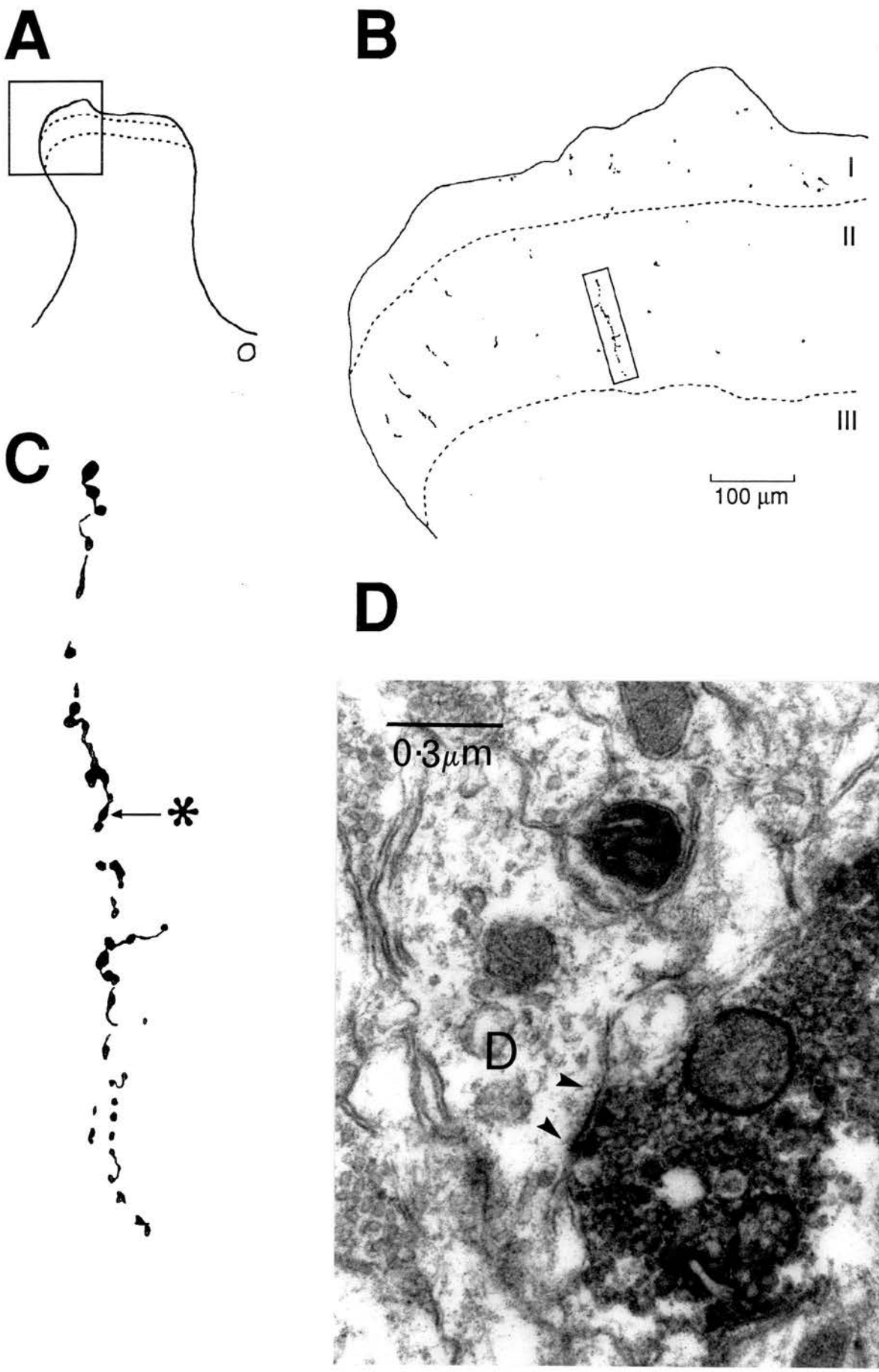

A
B
I
II
III
100 µm
C
*
D
0·3µm
D

Figure 9.

Correlated light and electron microscopic analysis of a TH-immunoreactive axon. **A.** Location of the axon in lamina II of the dorsal horn (■). **B.** Light microscopic appearance of the immunocytochemically stained axon. **C.** A low power electron micrograph of the same fibre. Note the presence of the two boutons marked 1 and 2. **D,E.** At higher magnification, the symmetric (Gray type II) synaptic junctions formed by these structures are resolved (between the arrowheads). Bouton 1 synapses with a large calibre dendrite (D), while the dendrite (D) contacted by bouton 2 is small.

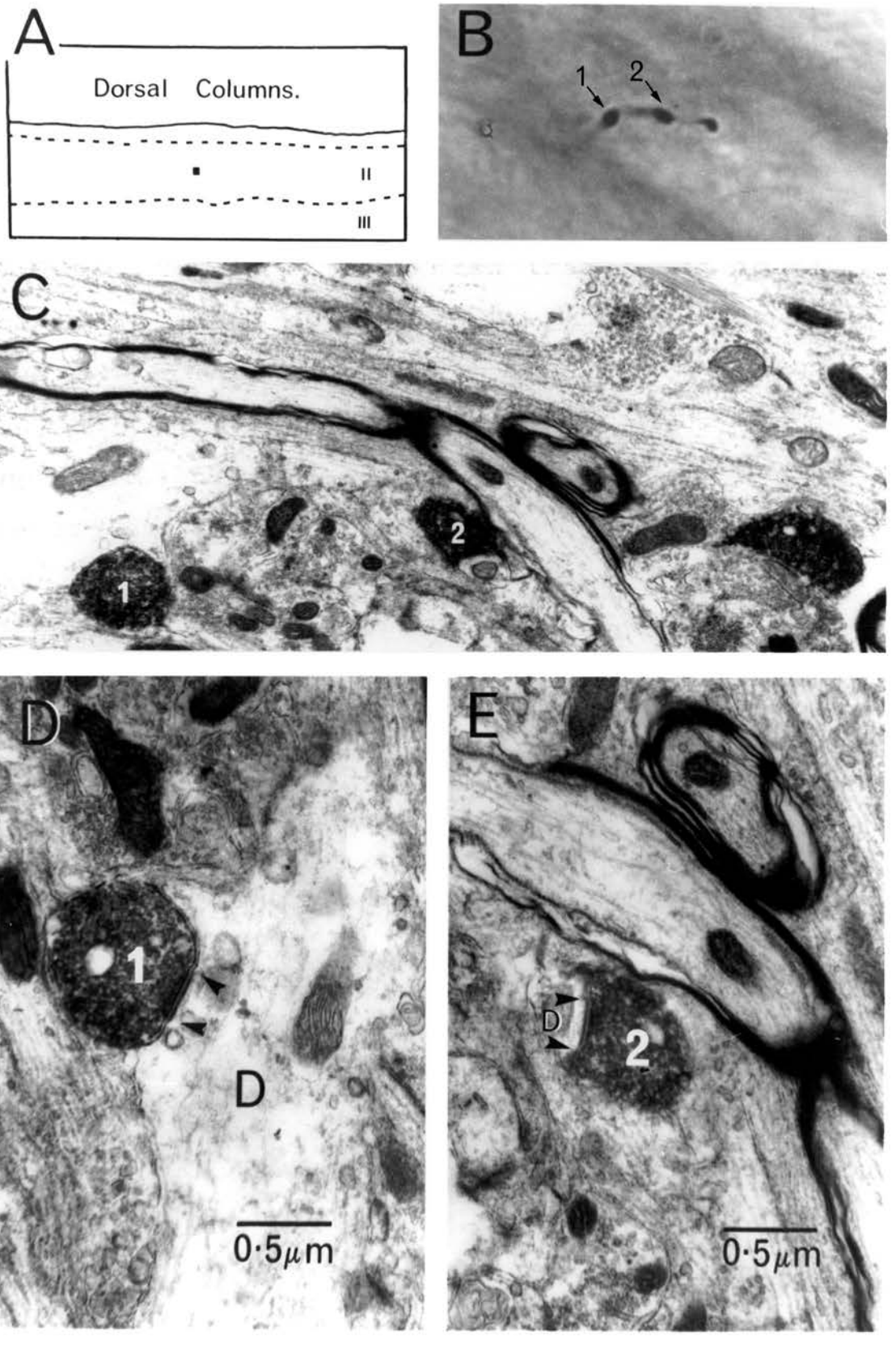

A
Dorsal Columns.
II
III
B
1
2
C
1
2
D
1
D
0·5μm
E
D
2
0·5μm

Figure 10.

Electron micrographs showing TH-immunoreactive terminals making symmetric synaptic junctions (between the arrow-heads) with small (**A,B**), medium (**C,D**) and large (**F**) dendritic profiles (D). The bouton in **F** is shown at lower magnification in **E** (*). At this magnification, the very large calibre of this dendrite (D) is revealed. Scale bars: A-D,F=0.25μm; E=2.0μm.

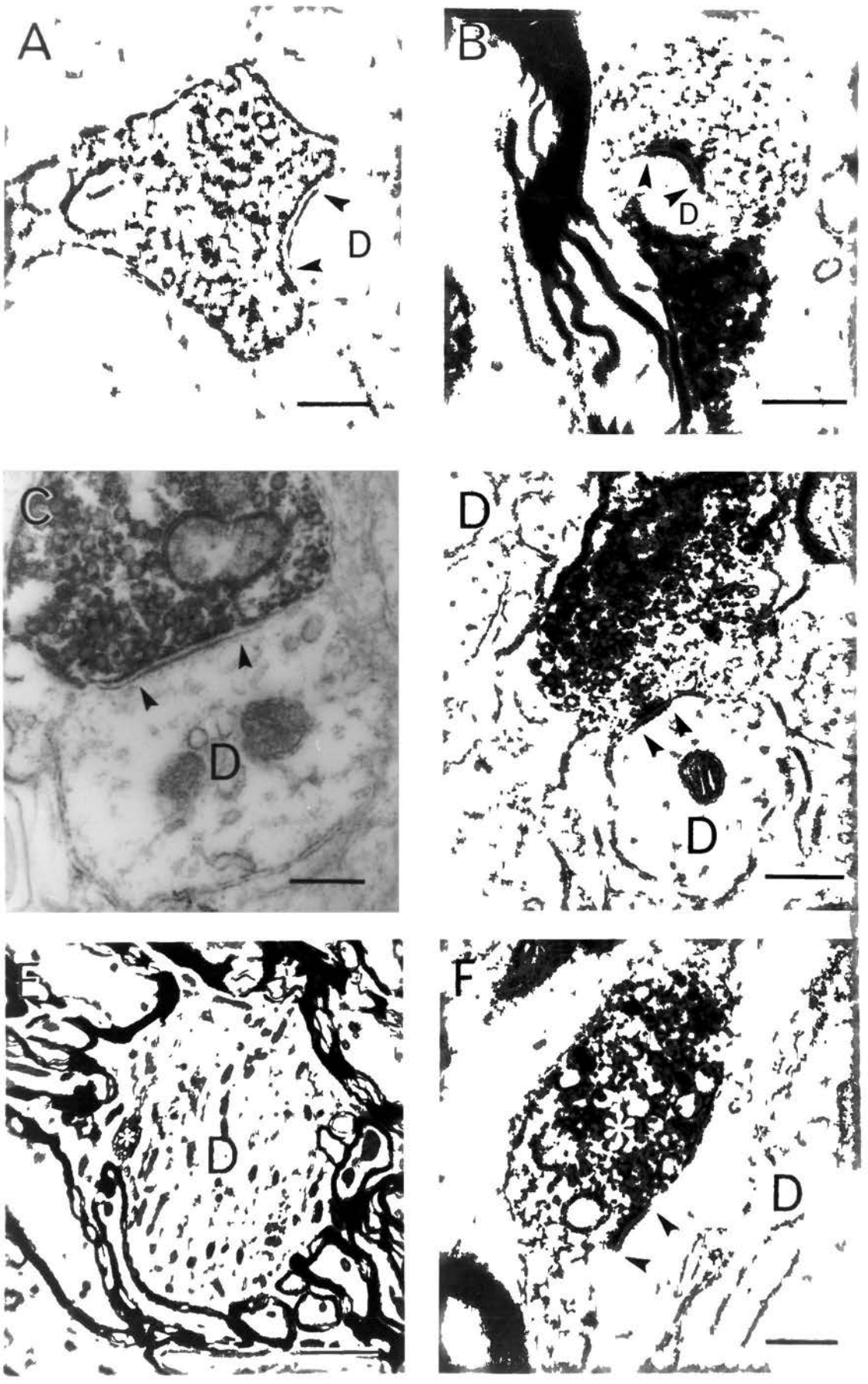

A
D
B
D
C
D
D
D
E
D
F
D

Figure 11.

Electron micrographs showing a TH-immunoreactive bouton forming a symmetric (Gray type II) synaptic junction with a large neuronal perikaryon in lamina IV. **A.** Location of the cell body within the dorsal horn (■). **B.** Low power electron micrograph of the bouton (B) apposed to the large soma. Note the presence of the nucleus (Nuc). **C.** At higher magnification the prominant active zone is revealed (between the arrowheads).

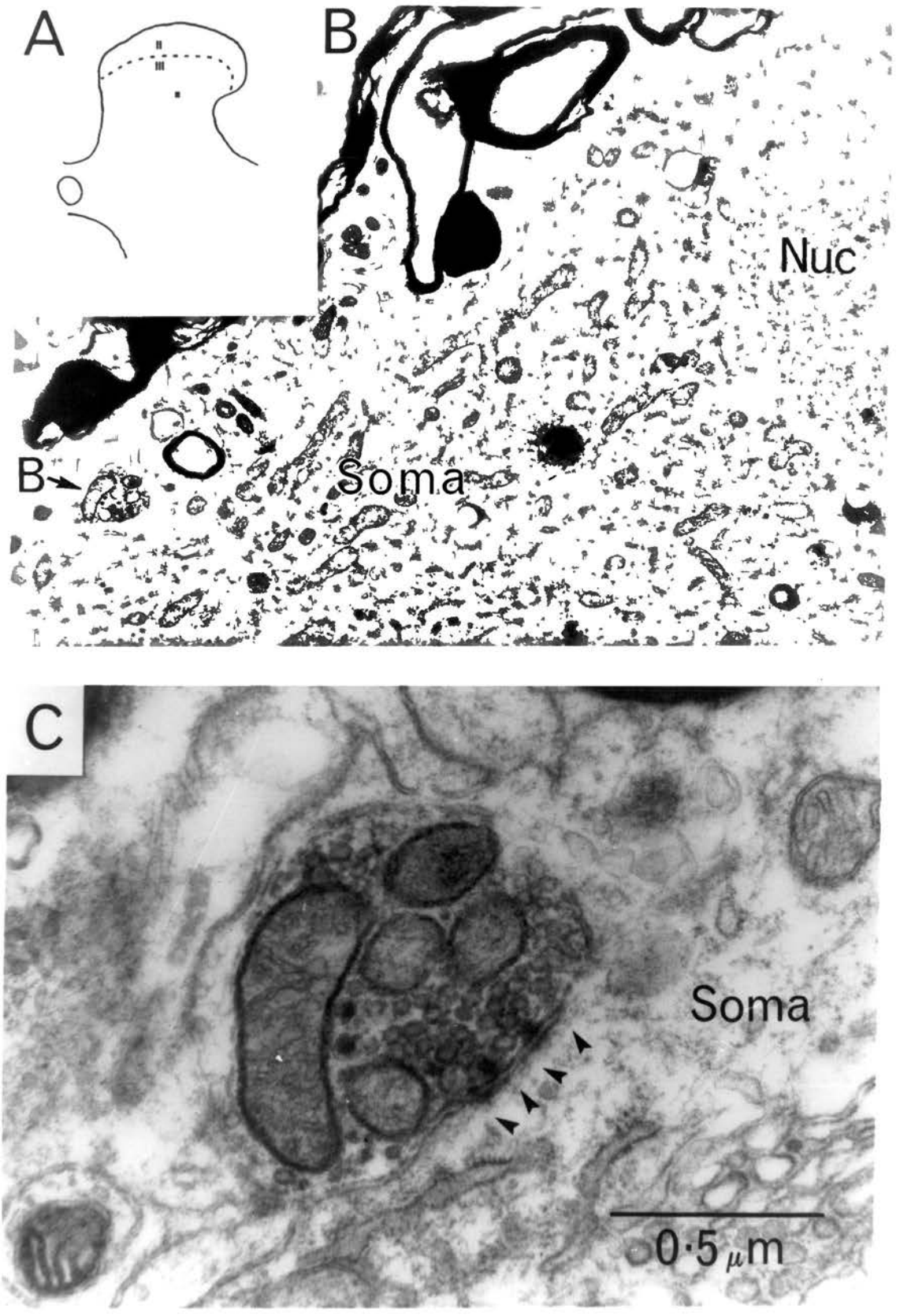

A
B
Nuc
B
Soma
C
Soma
0·5 μm

Figure 12.

A. Frequency distribution histogram of TH-immunolabelled bouton diameters. The majority (84%) of profiles fall within the range 0.6-1.2μm (mean diameter=0.95μm).

B. Frequency distribution histogram of postsynaptic dendritic diameters. TH-immunoreactive boutons synapse most commonly with medium-sized (0.5-2.0μm diameter) dendrites. Large calibre dendritic shafts (diameter >2.0μm) also receive input from TH-immunoreactive axonal endings, as do very small (<0.5μm diameter) dendrites.

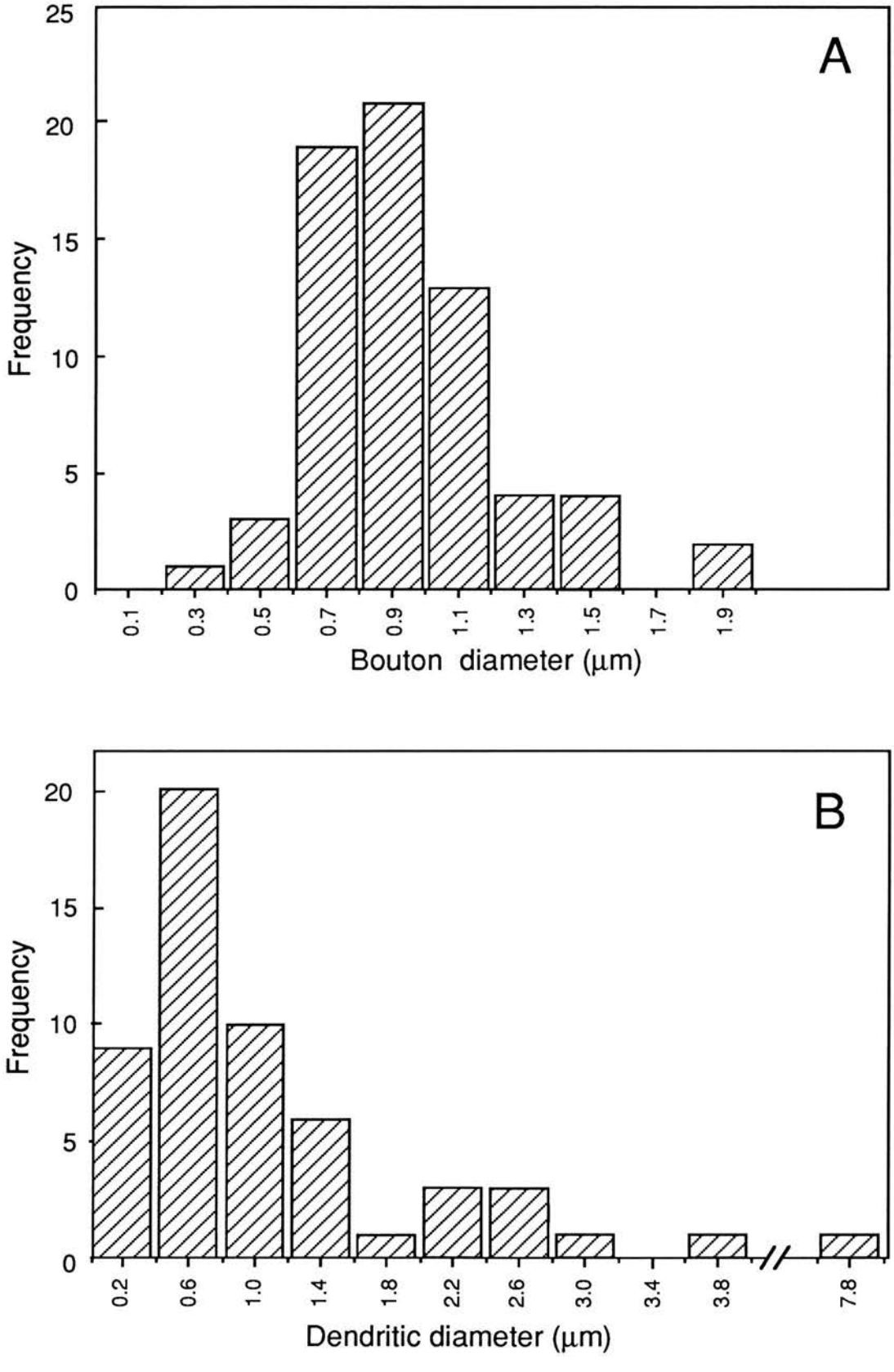

A
Frequency
25
20
15
10
5
0
0.1
0.3
0.5
0.7
0.9
1.1
1.3
1.5
1.7
1.9
Bouton diameter (μm)
B
Frequency
20
15
10
5
0
0.2
0.6
1.0
1.4
1.8
2.2
2.6
3.0
3.4
3.8
7.8
Dendritic diameter (μm)

Figure 13.

Correlated light and electron microscopic analysis of a DBH-immunoreactive axon (Triton X-100 treated material). **A.** Position of the axon in lamina II of the dorsal horn (■). **B.** Light microscopic appearance of the axon. Boutons 1-5 are illustrated in the electron micrographs **C-G.** These boutons formed symmetric (Gray type II) axo-dendritic synaptic junctions. Active zones (between the arrowheads) of boutons 2-5 and their postsynaptic dendrites (D) are shown in **D-G**. Bouton 1 additionally formed a punctum adhaerens (arrowheads) with a dendrite, (D), (**C**). Synaptic contacts were observed upon small- (<0.5µm diameter: **D,F**) and medium-sized (0.5-2.0µm diameter: **E**) dendrites, as well as dendritic spines (**G**). In **G**; B.V.= blood vessel.

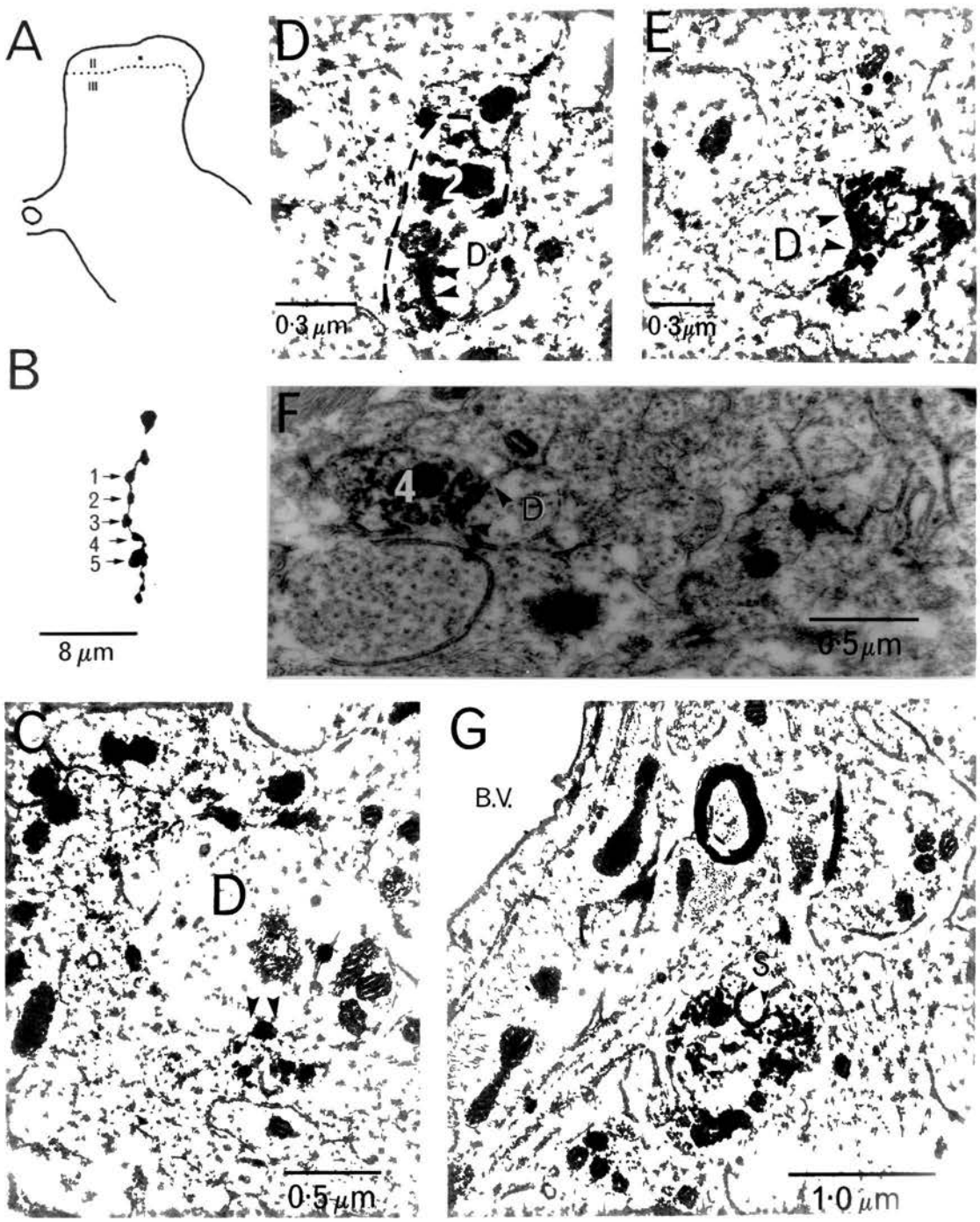

A
II
III
B
1
2
3
4
5
8 μm
D
D
0·3 μm
E
D
0·3 μm
F
4
D
0·5 μm
C
D
0·5 μm
G
B.V.
S
1·0 μm

Figure 14.

Correlated light and electron microscopic analysis of a DBH-immunoreactive axon (Triton X-100 treated material). This fibre was located at the lamina I-II border (■) (**A**), and its light microscopic appearance is shown in **B**. Boutons 1 and 2 are illustrated in the electron micrographs **C–F**. **C**. A low power electron micrograph showing close apposition between bouton 1 and a neuronal perikaryon (P), nucleus, N. **D**. At higher magnification a symmetric synaptic junction is revealed (between the arrowheads), n, Nissl substance. **E,F**. Serial sections of the axo-dendritic synapse formed by bouton 2. The morphology of this junction changed as it was followed through serial sections: in micrograph **E** it appears symmetric, whereas in **F** it is asymmetric (Gray type I).

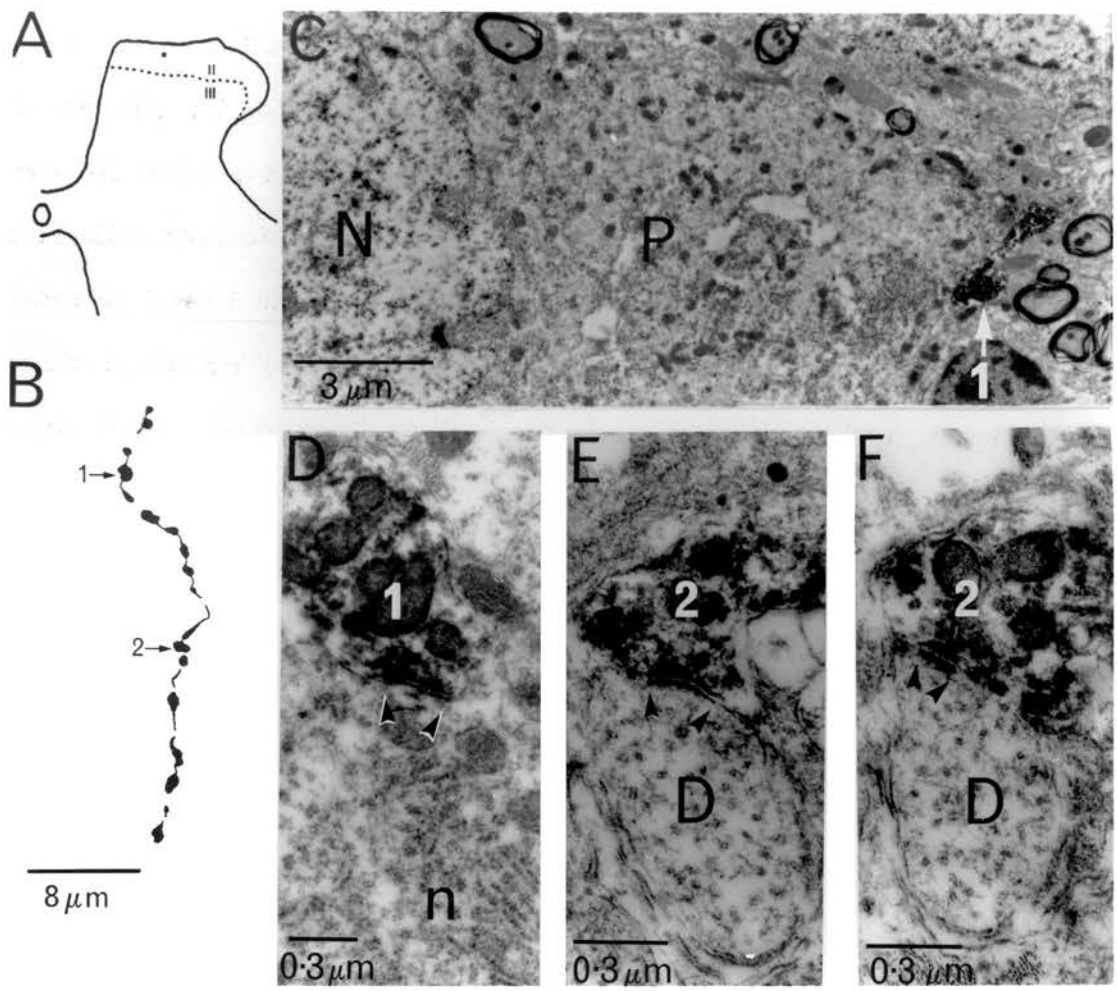

A
II
III
o
B
1
2
8 μm
C
N
P
3 μm
1
D
1
n
0·3 μm
E
2
D
0·3 μm
F
2
D
0·3 μm

Figure 15.

Immunocytochemical controls.

Both TH (A,B) and DBH (F,G) antisera labelled neuronal cell bodies in the locus ceoruleus (A6 noradrenergic cell group). In contrast, only TH antibodies labelled nerve fibres in the striatum (C,D): the DBH antiserum produced a negative result in this tissue (H,I). Spinal cord sections which had been incubated in a medium from which the TH (E) & DBH (J) antiserum had been omitted displayed no immunolabelling.

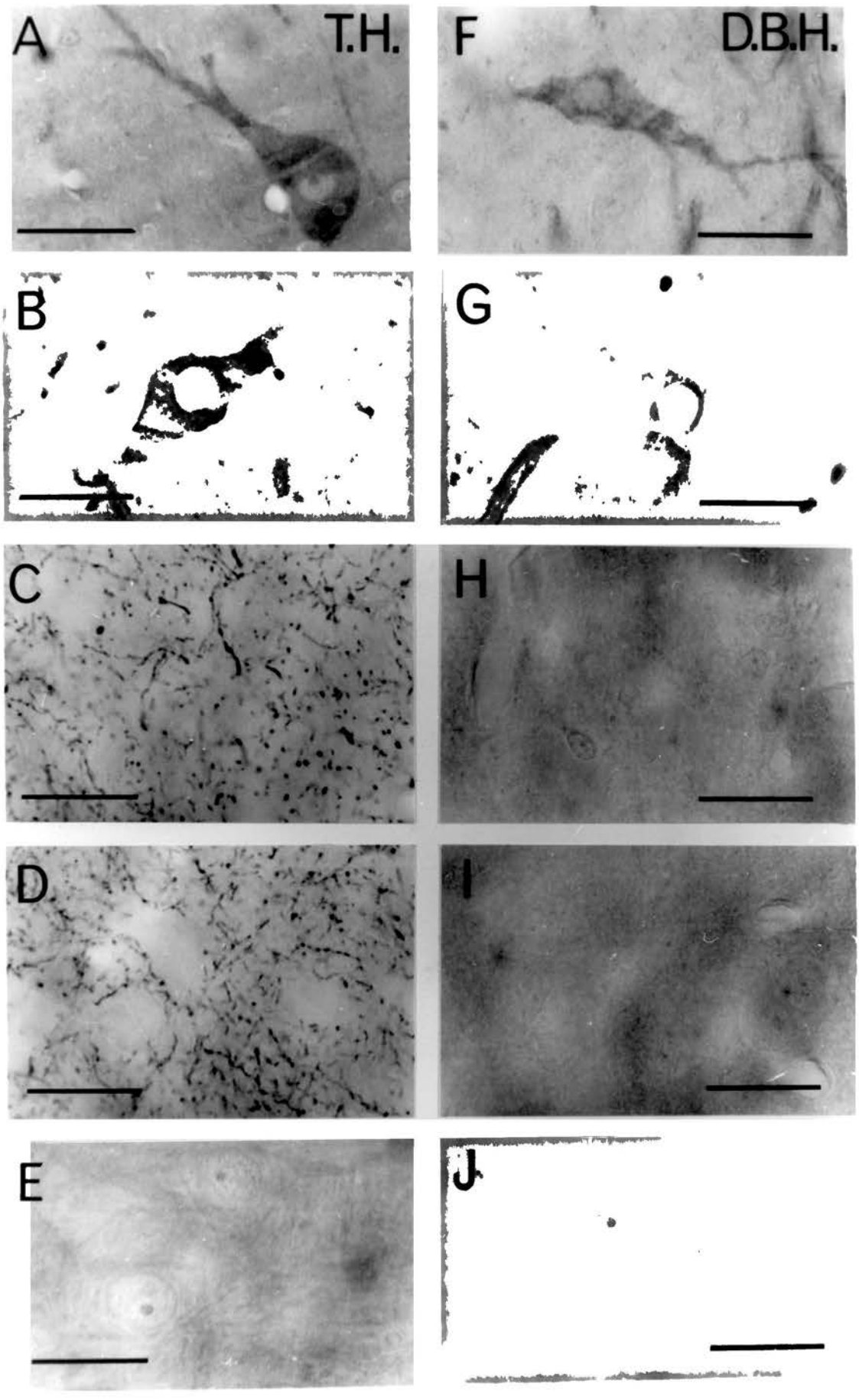

A
T.H.
F
D.B.H.
B
G
C
H
D
I
E
J

Figure 16.

Drawings of thoracic (A-C) and cervical (D,E) spinal cord illustrating the area of destruction associated with the dorsal column implant sites (A-D) and the cervical DLF lesion (E). The drawings show the maximum extent of each lesion. The hatched areas in A-D indicate the spread of HRP around the implant site.

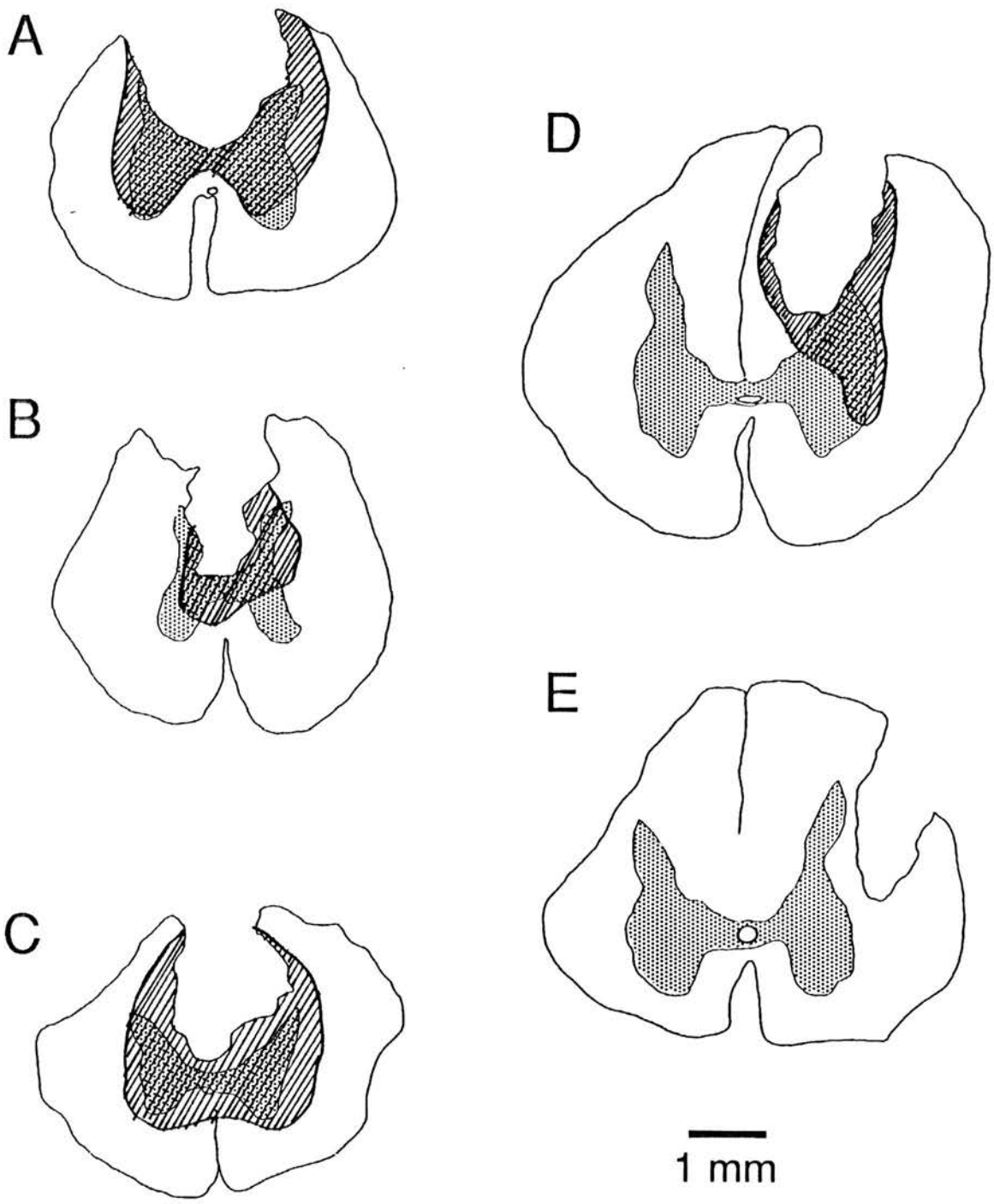

A
B
C
D
E
1 mm

Figure 17.

The laminar locations of 44 retrogradely labelled neurons, found within laminae III-V, which received contacts from CA-containing axons. The position of each cell body has been placed on a standard dorsal horn according to its relative position both dorsoventrally and mediolaterally in its own dorsal horn. Note that most of the innervated cells (70%) were found within the medial half of the dorsal horn. Five labelled cells receiving contacts from CA-containing boutons which were confirmed ultrastructurally to be synaptic associations are indicated (*).

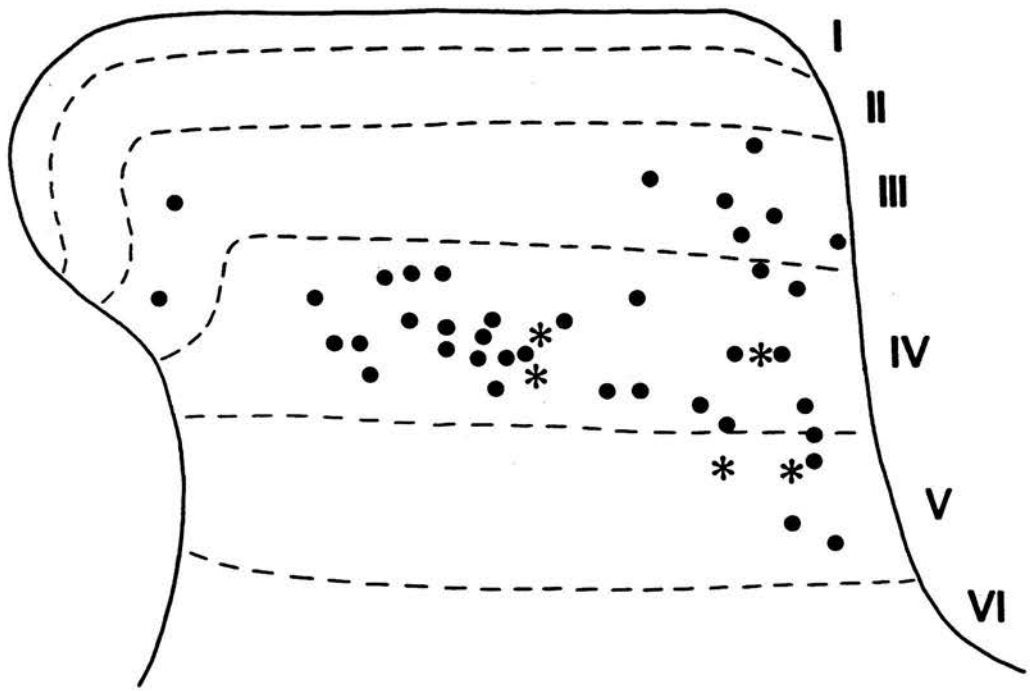

I
II
III
IV
V
VI

Figure 18.

A,C,E. Drawings of 3 retrogradely labelled PSDC neurons observed following HRP implantation into thoracic (T10-T12) spinal cord. The arrows point to TH-immunoreactive (A,C) or DBH-immunoreactive (E) varicosities which are also shown in the corresponding light micrographs (B,D,F).

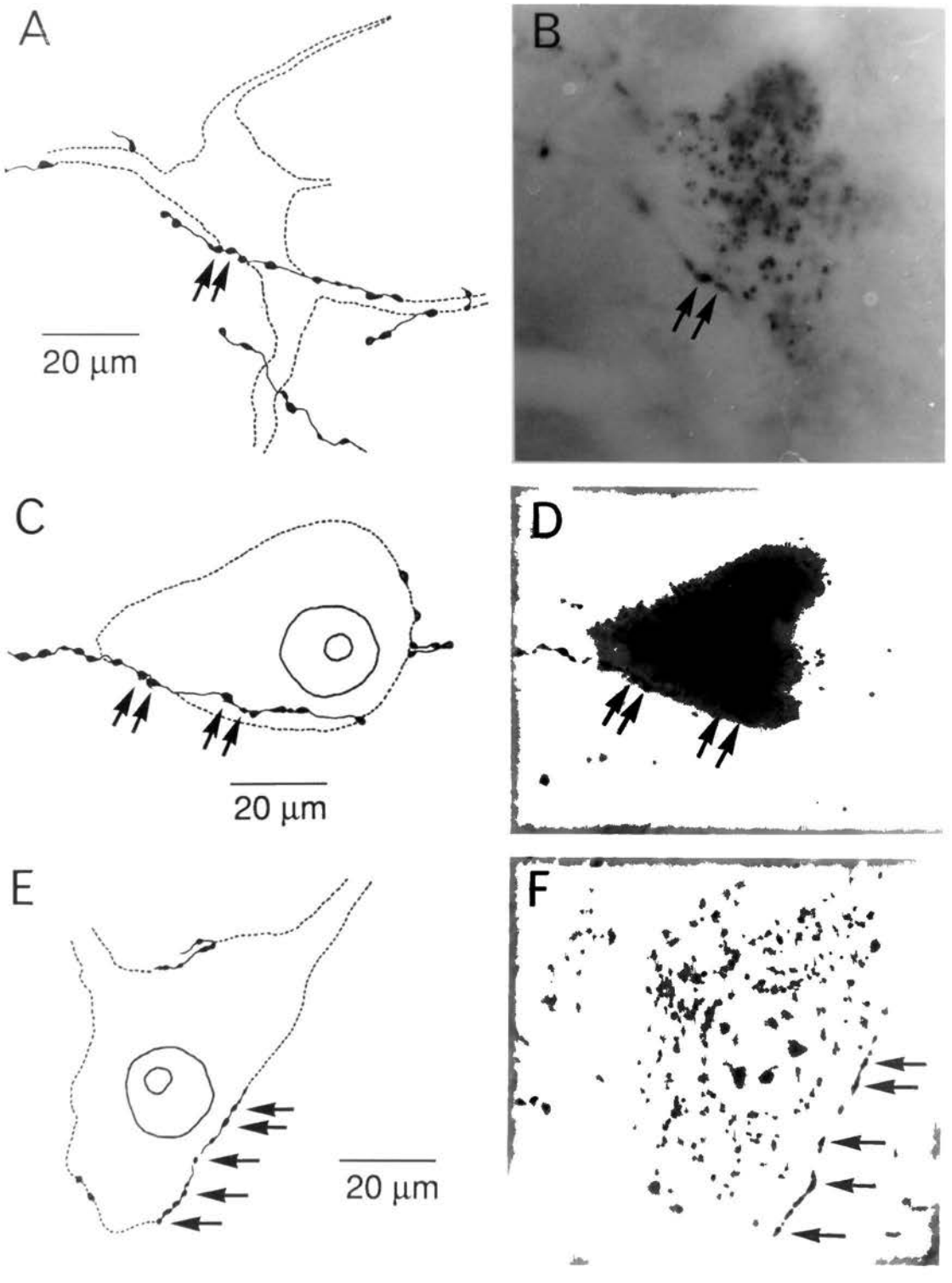

A
20 µm
B
C
20 µm
D
E
20 µm
F

Figure 19.

A,C. Drawings of 2 retrogradely labelled PSDC neurons observed following implantation of HRP into cervical (C2-C3) spinal cord, which were innervated by axons immunoreactive for DBH. The arrows point to varicosities which are also shown in the corresponding light micrographs **(B,D)**.

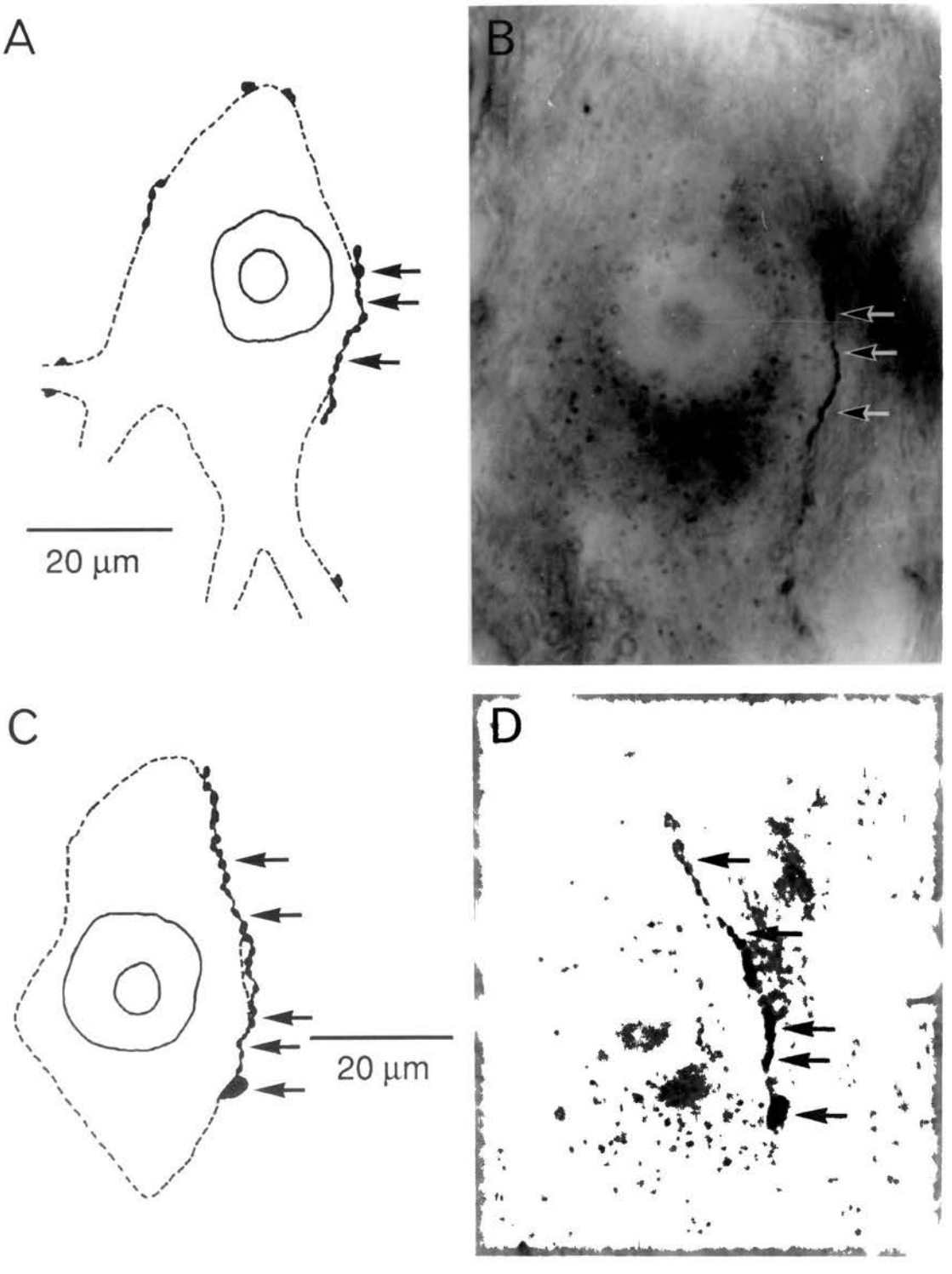

A
20 µm
B
C
20 µm
D

Figure 20.

Correlated light and electron microscopic analysis of a PSDC neuron from lamina IV which received a TH-immuno-reactive axo-somatic contact. **A**. A drawing of the retrogradely labelled neuron and immunostained bouton (B). **B**. Electron micrograph of the neuron. The bouton (B) directly apposes the perikaryon (P) which contains a granule of retrogradely transported HRP (*). **C**. At higher magnification the synaptic junction formed by this bouton is revealed (between the arrowheads).

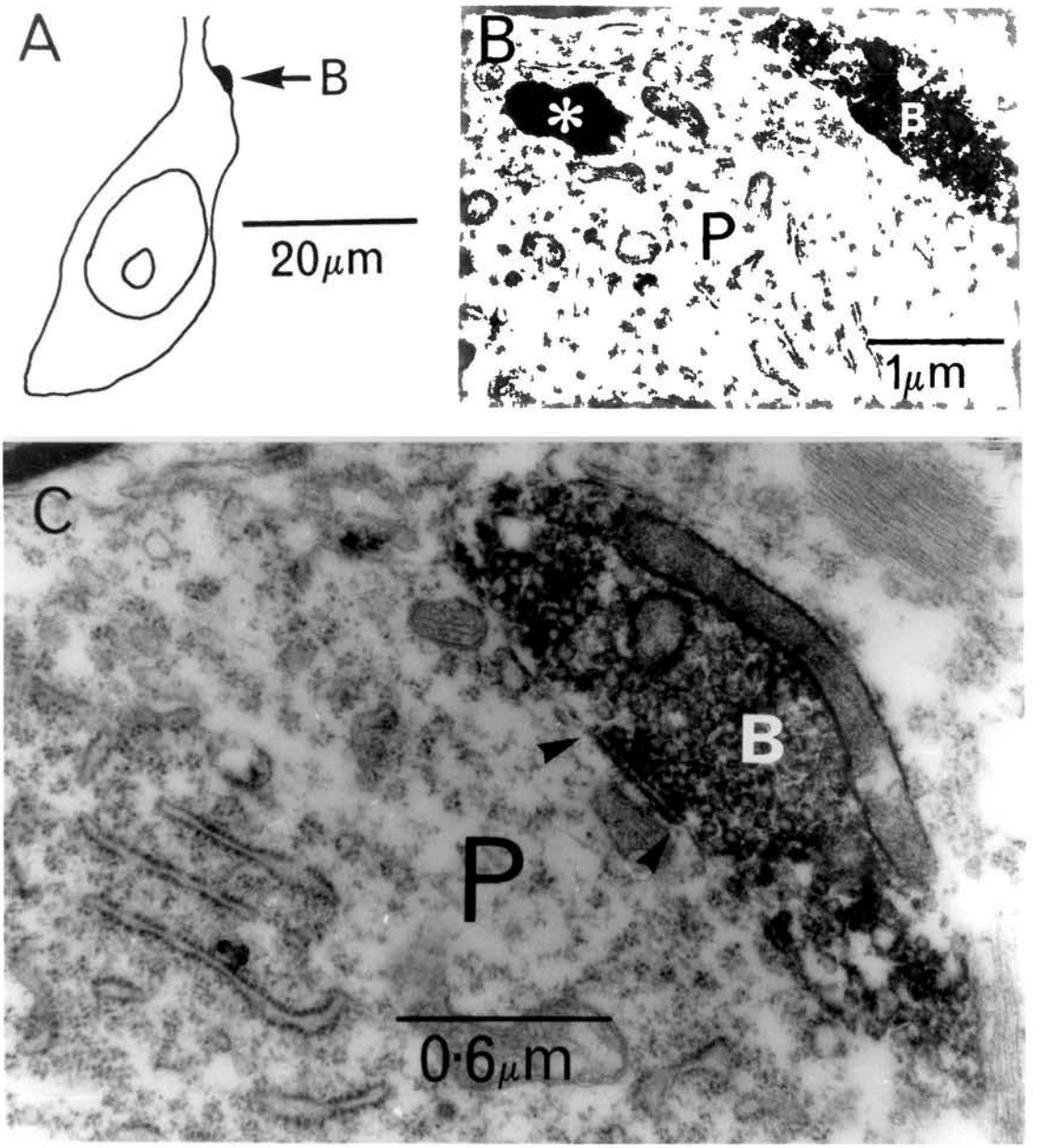

A
B
20μm
B
*
B
P
1μm
C
B
P
0·6μm

Figure 21.

Correlated light and electron microscopic analysis of a PSDC neuron receiving a TH-immunoreactive axo-dendritic contact. **A.** Light micrograph of the retrogradely labelled neuron and the immunostained bouton (B) contacting it. Note the presence of HRP granules within the cell body and proximal dendrites. The area enclosed by the box is shown at a higher magnification in the electron micrograph **C.** Note the granules of retrograde HRP (arrows) within the dendrite (D) and the contact from the immunostained bouton (B). **B** shows the location of the cell body in lamina V. At higher magnification (**D**) the synaptic junction formed by this bouton is revealed (between the arrowheads).

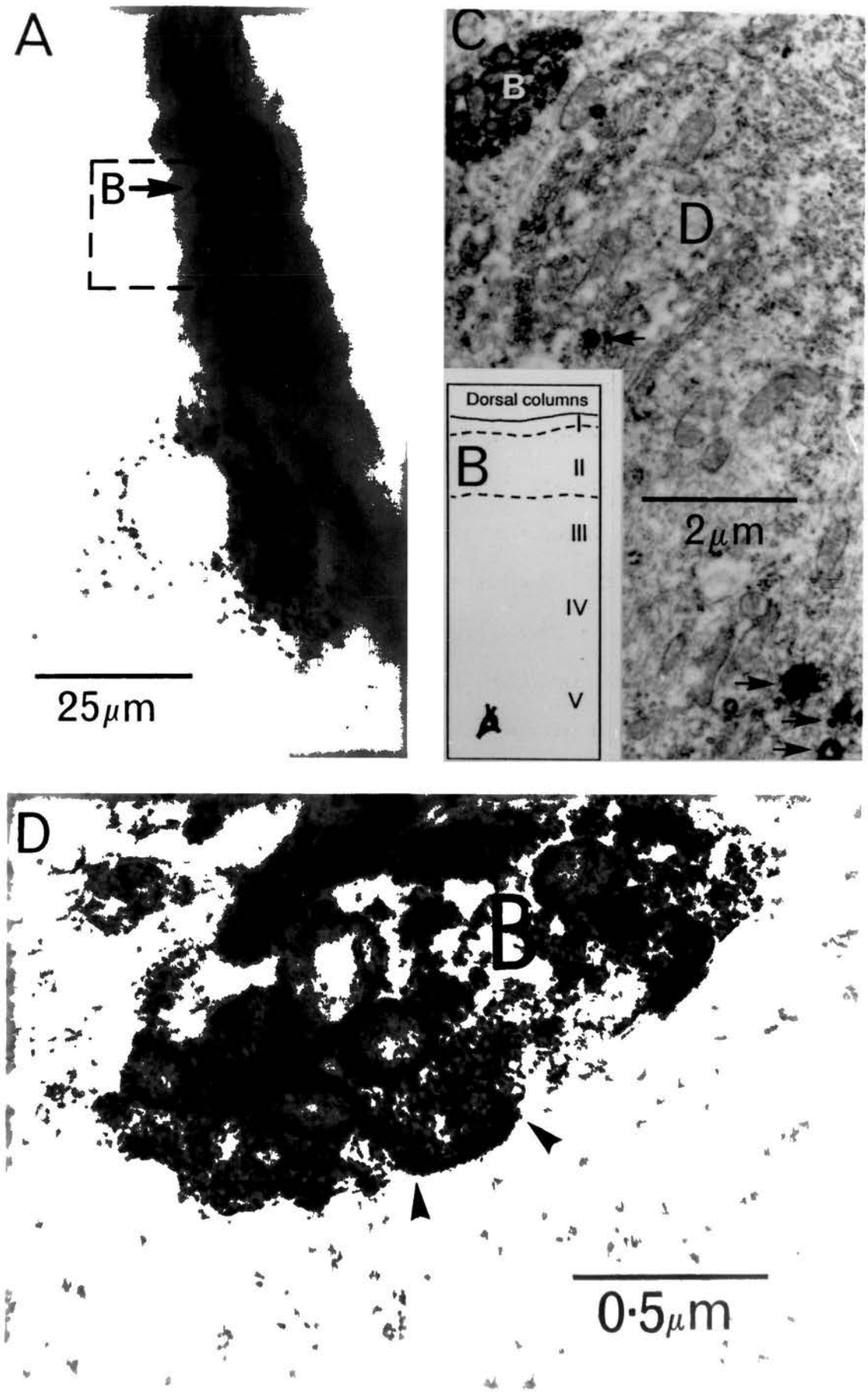

A
B
25μm
C
B
D
Dorsal columns
B
II
III
IV
V
2μm
D
B
0·5μm

Figure 22.

Drawings of cervical spinal cord illustrating the area of destruction associated with the DLF implant sites (**A,C**) and the dorsal column lesions (**B,D**). The drawings show the maximum extent of each lesion. The hatched areas in **A** and **C** indicate the spread of HRP around the implant site.

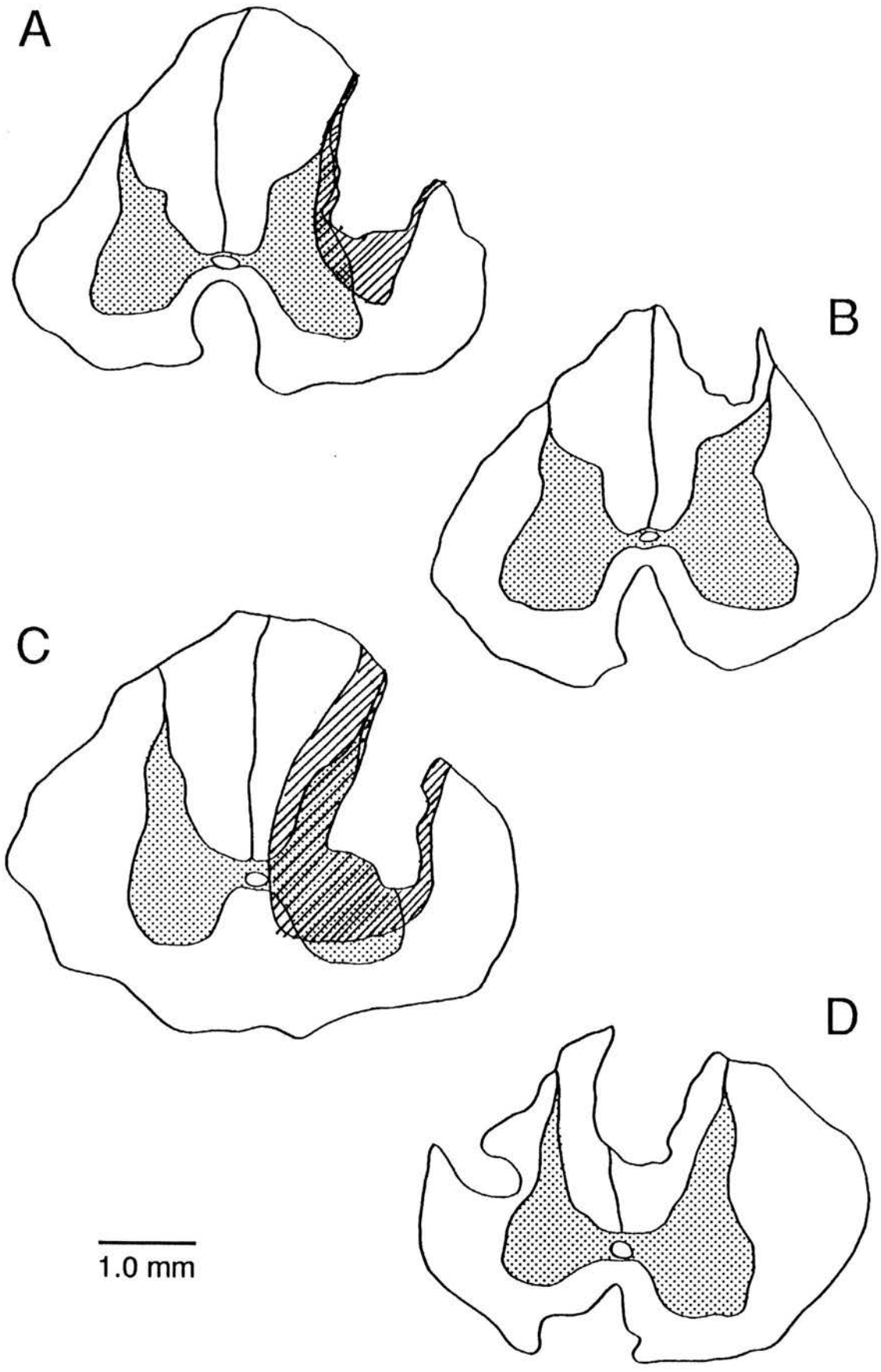

A
B
C
D
1.0 mm

Figure 23.

Light micrographs illustrating the relationships between retrogradely labelled SCT cells and DBH- (A,D) or TH- (B,C) immunostained axons in laminae III-V of the dorsal horn. Most HRP-labelled neurons (87%) did not receive contacts from CA-containing axons (A), although there was often numerous immunostained axons close by. However, 13% of labelled cells had TH- (B,C) or DBH- (D) positive terminals (arrowheads) directly apposed to their proximal dendrites (B) or somata (C,D). Most immunolabelled contacts were single (B-D), but occasionally a varicose axon was seen to follow the curvature of a dendrite or somata. Scale bars=20μm.

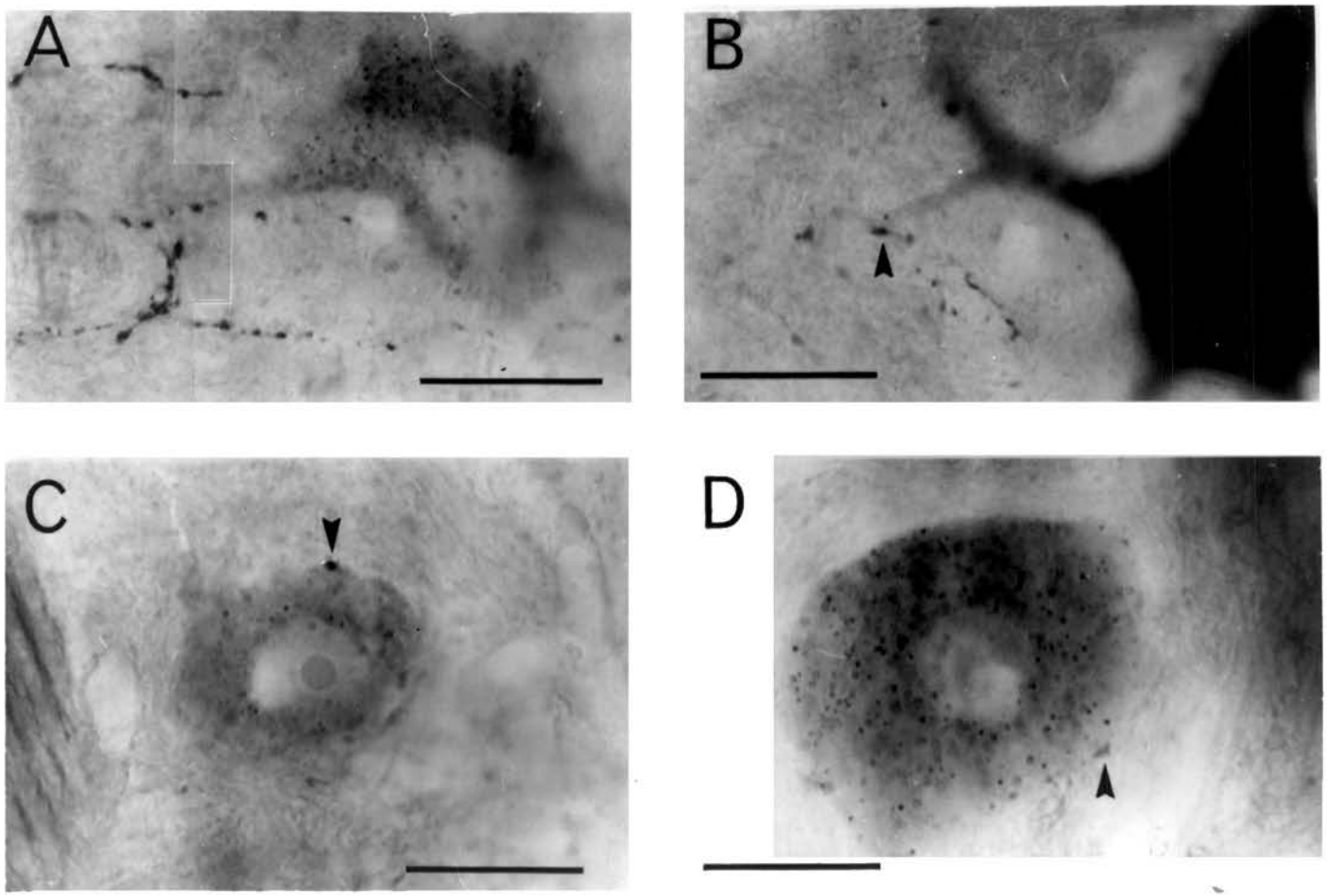

A
B
C
D

Figure 24.

Distribution of TH-immunoreactivity in rostral (**A**), mid (**B**) and caudal (**C**) regions of the LCN. Numerous punctate varicosities and short axons were present throughout the nucleus, and some of these contacted neuronal somata (shaded structures). Some of these cells were large (soma diameter=50µm). The insets show the position of the LCN relative to the dorsal horn (DH).

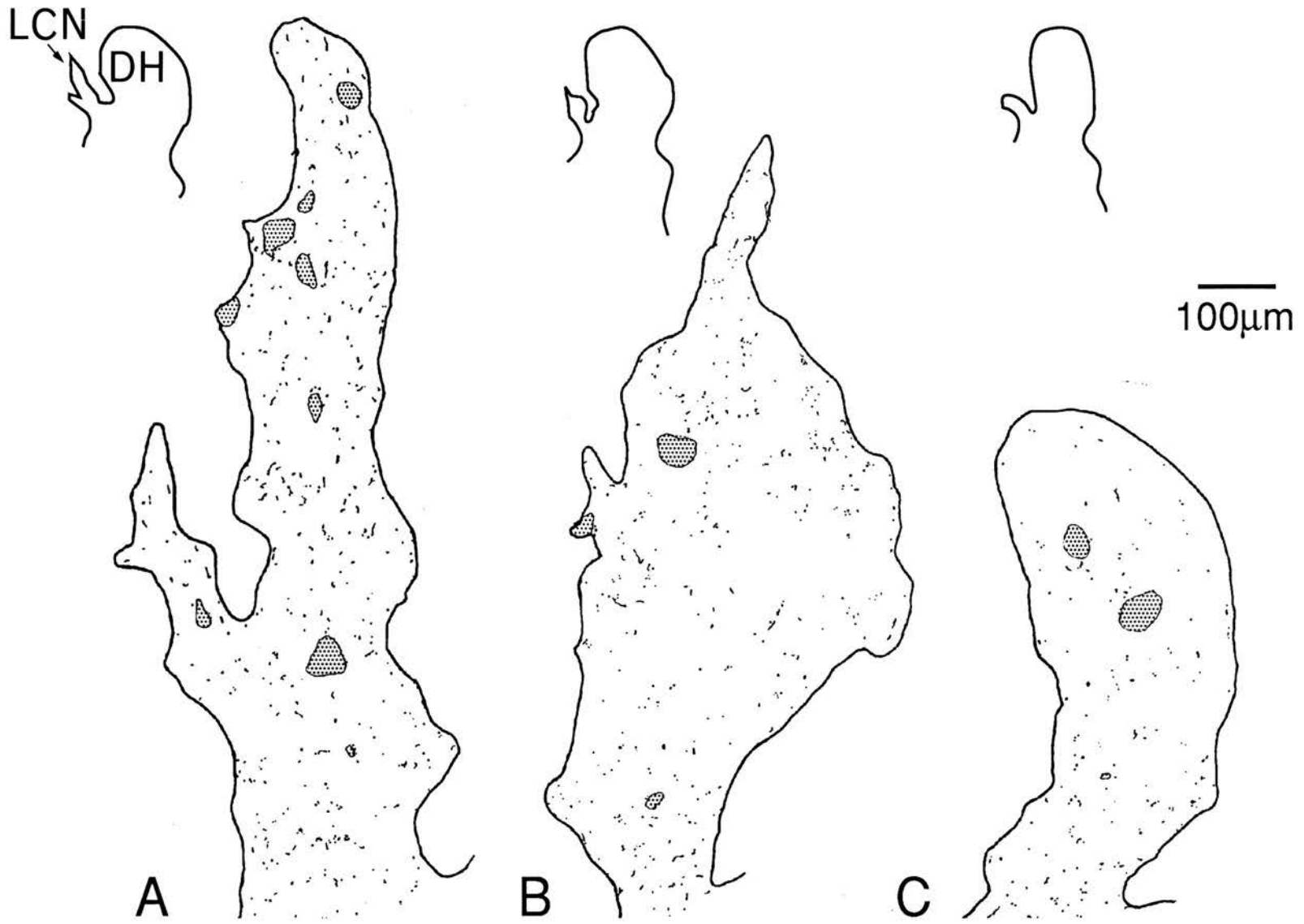

LCN
DH
100μm
A
B
C

Figure 25.

Light microscopic appearance of TH-IR varicose axons (arrowheads) in the feline LCN. Long strands of TH-positive axons were some times seen (**A**), but most profiles appeared as single varicosities (**B**) or short axons with few *en passant* swellings (**B–D**). Scale bars: A,B=10μm; C,D=5μm.

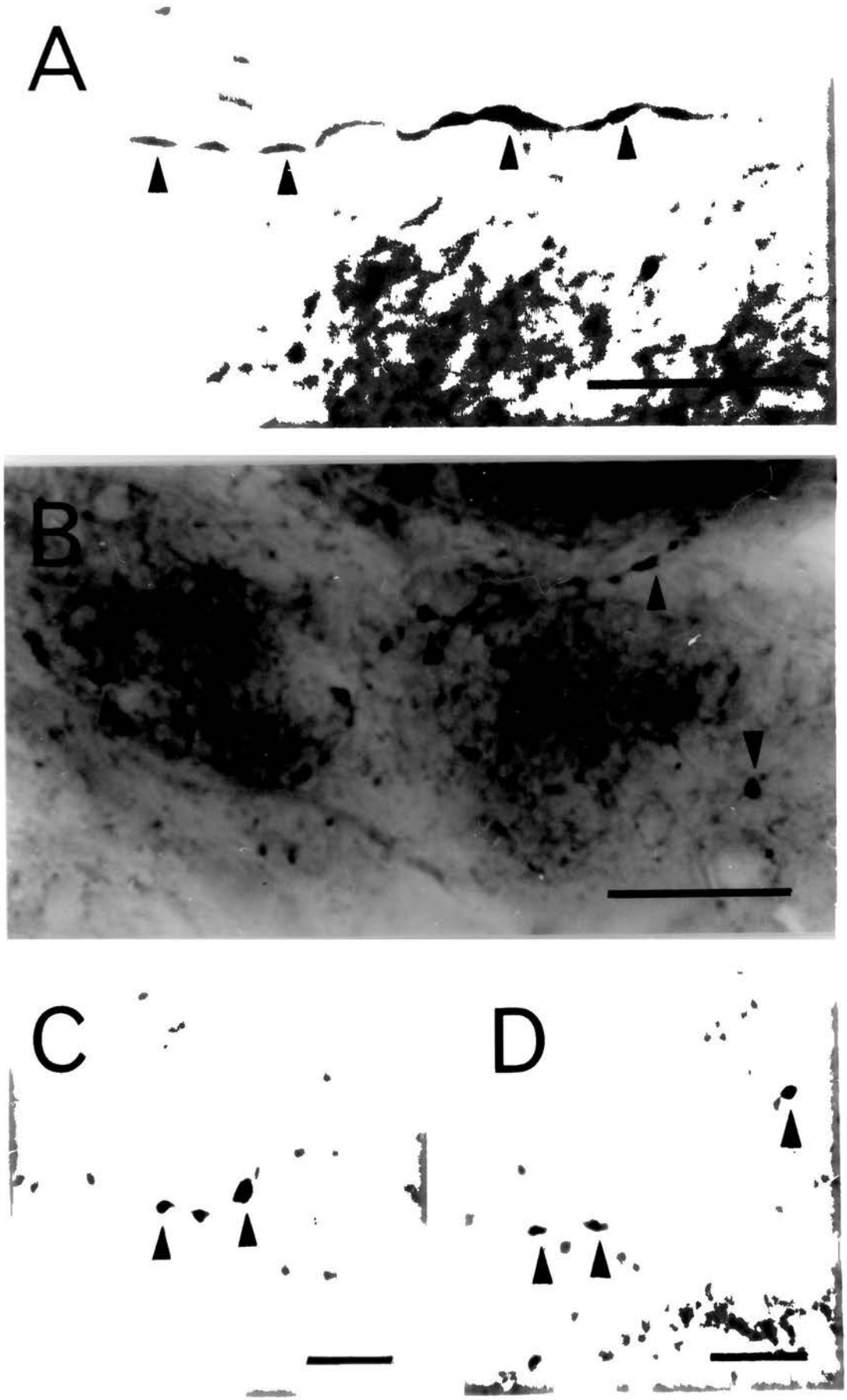
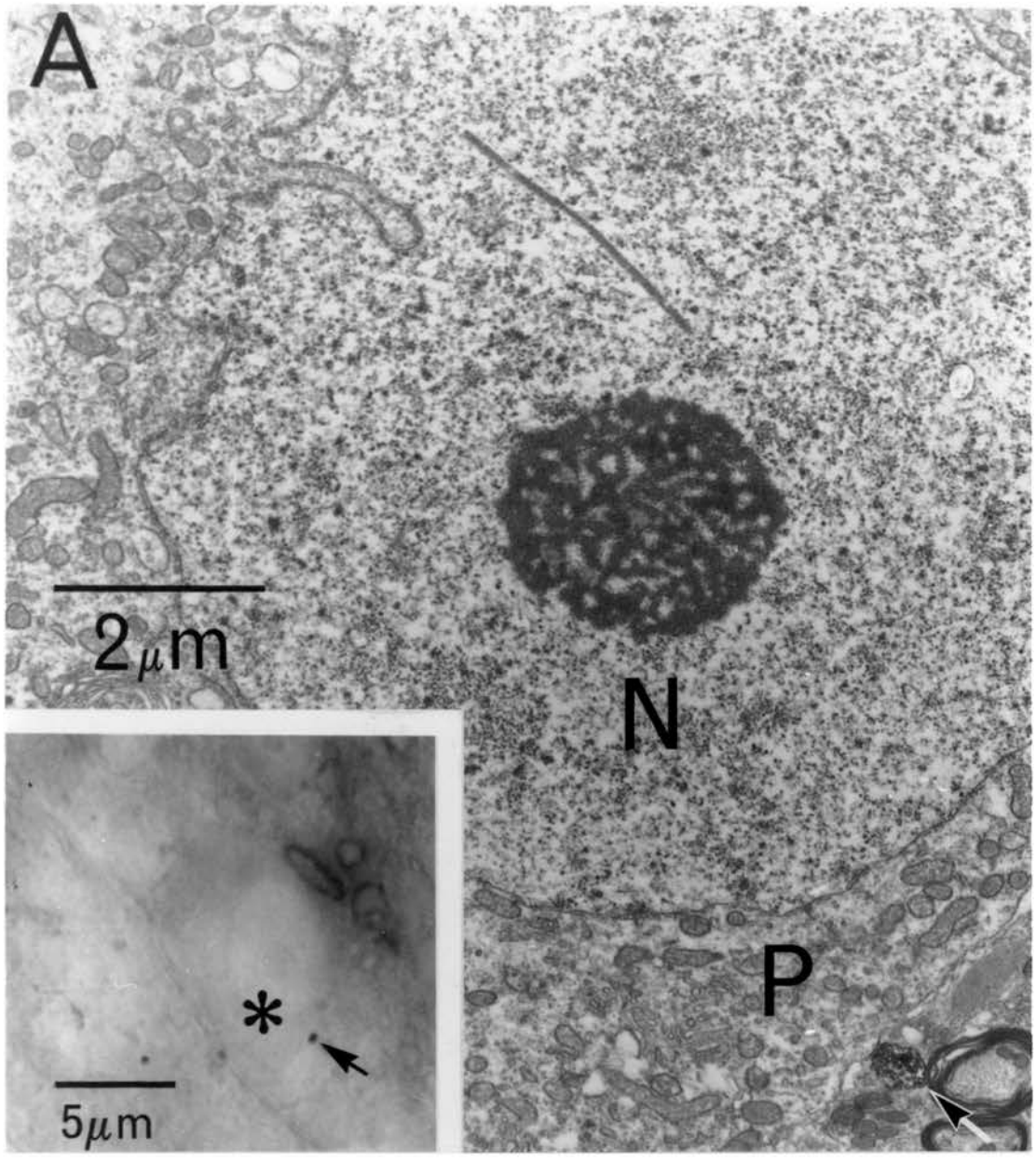

A
B
C
D

Figure 26,

Correlated light and electron microscopic analysis of a TH-IR axon terminal which formed an axo-somatic synaptic junction with a small LCN neuron (10μm diameter) in the medial part of the nucleus. (**A**) shows the ultrastructural appearance of the LCN neuron. Note the presence of the small TH-IR bouton (arrow) in contact with the perikaryon (P; N=nucleus). The light microscopic appearance of this arrangement is shown in the inset. The small TH-positive terminal (arrow) is adjacent to the neuronal cell body (*). At high magnification (**B**), the symmetric synaptic junction formed between the TH-IR bouton and the perikaryon (P) can be seen (arrowheads). This synapse is shown in serial section in the inset.


A
2μm
N
P
5μm

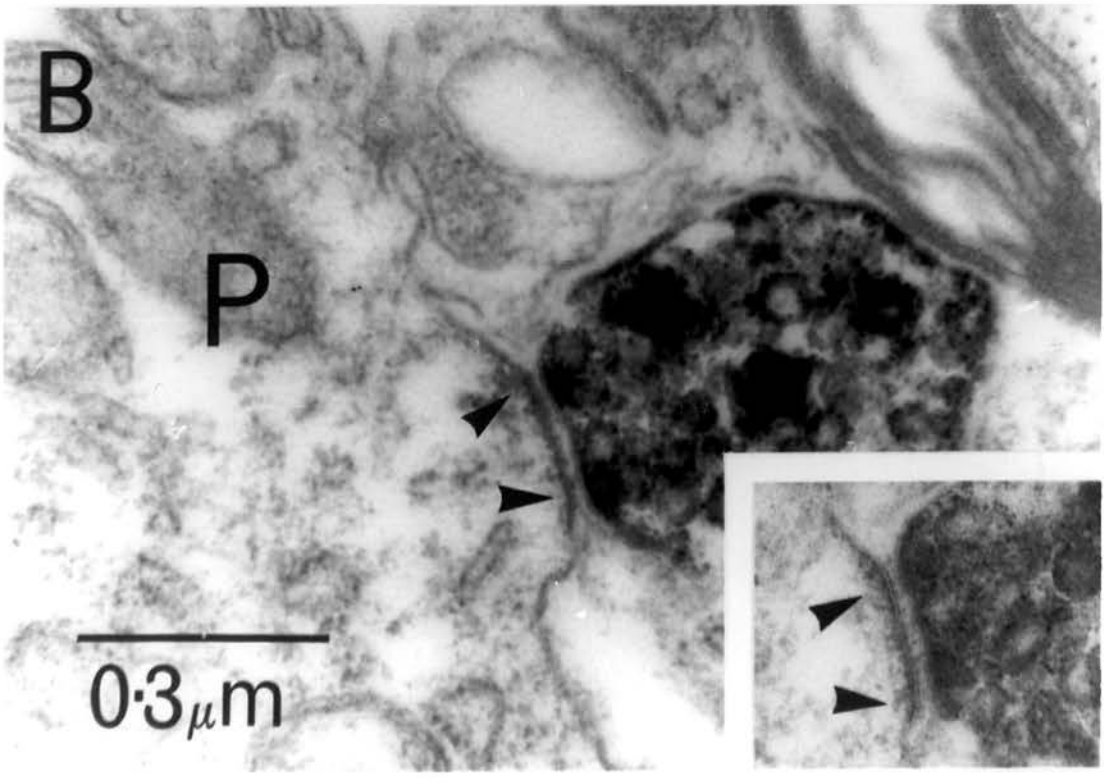

B
P
0·3μm

Figure 27.

Correlated light and electron microscopic analysis of TH-IR boutons in the lateral LCN. (**A**) shows the position of the terminals in the LCN (circle) and a camera lucida drawing of their light microscopic appearance is illustrated in the inset. The boutons marked a-d are illustrated in the electron micrographs B-F. (**B-D**) Three of these boutons (a-c) formed symmetric synaptic junctions (arrowheads) with dendrites (Den). (**E**) The fourth bouton (d) (arrow) contacted a large (>20μm diameter) LCN neuron (P=perikaryon; N=nucleus). At higher magnification (**F**), the symmetric synaptic junction formed by this bouton can be seen (between the arrowheads).

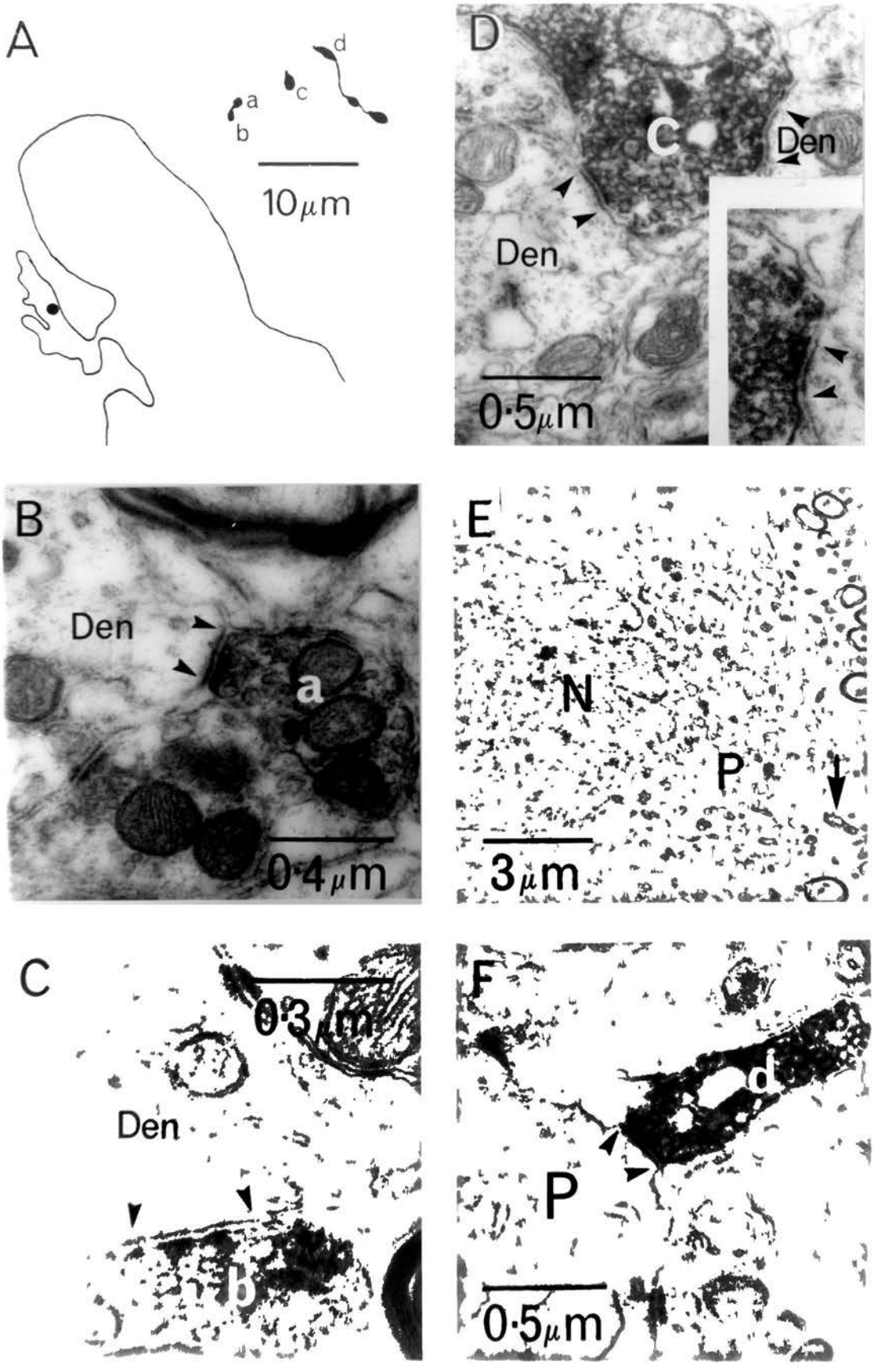

A
a
b
c
d
10μm
D
C
Den
Den
0·5μm
B
Den
a
0·4μm
E
N
P
3μm
C
0·3μm
Den
b
F
d
P
0·5μm

Figure 28.

Electron micrographs showing TH-IR boutons making symmetric axo-dendritic synaptic junctions (arrowheads) in the LCN. Small dendrites (**A**) and spine-like processes (**B**) were postsynaptic to CA-containing axon terminals, as were medium-sized (**C,D**) and large (**E**) dendrites. The bouton in (**E**) is shown at higher magnification in (**F**).

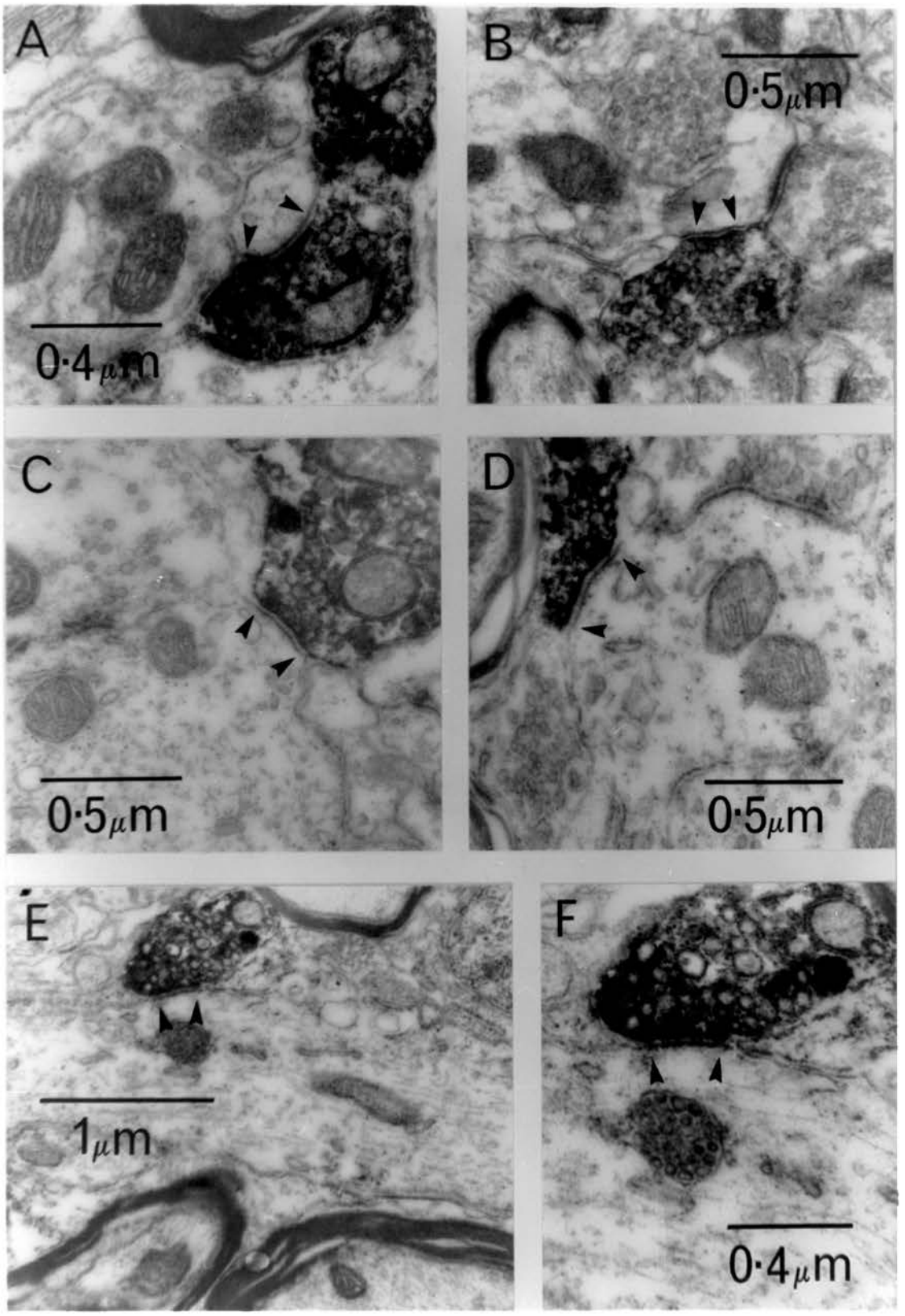

A
0·4μm
B
0·5μm
C
0·5μm
D
0·5μm
E
1μm
F
0·4μm

Figure 29.

(A) Frequency distribution histogram of immunolabelled bouton diameters. The majority of profiles (73%) fell within the range 0.6-1.2μm (mean diameter=0.77μm).

(B) Frequency distribution histogram of postsynaptic dendritic diameters. Immunolabelled boutons synapsed most commonly with medium sized (0.5-2.0μm diameter) dendrites (91% of axo-dendritic synapses). Large dendrites (>2.0μm diameter) also received input from TH-IR axonal endings (3% of axo-dendritic synapses), as did small (<0.5μm diameter) dendrites (6% of axo-dendritic synapses).

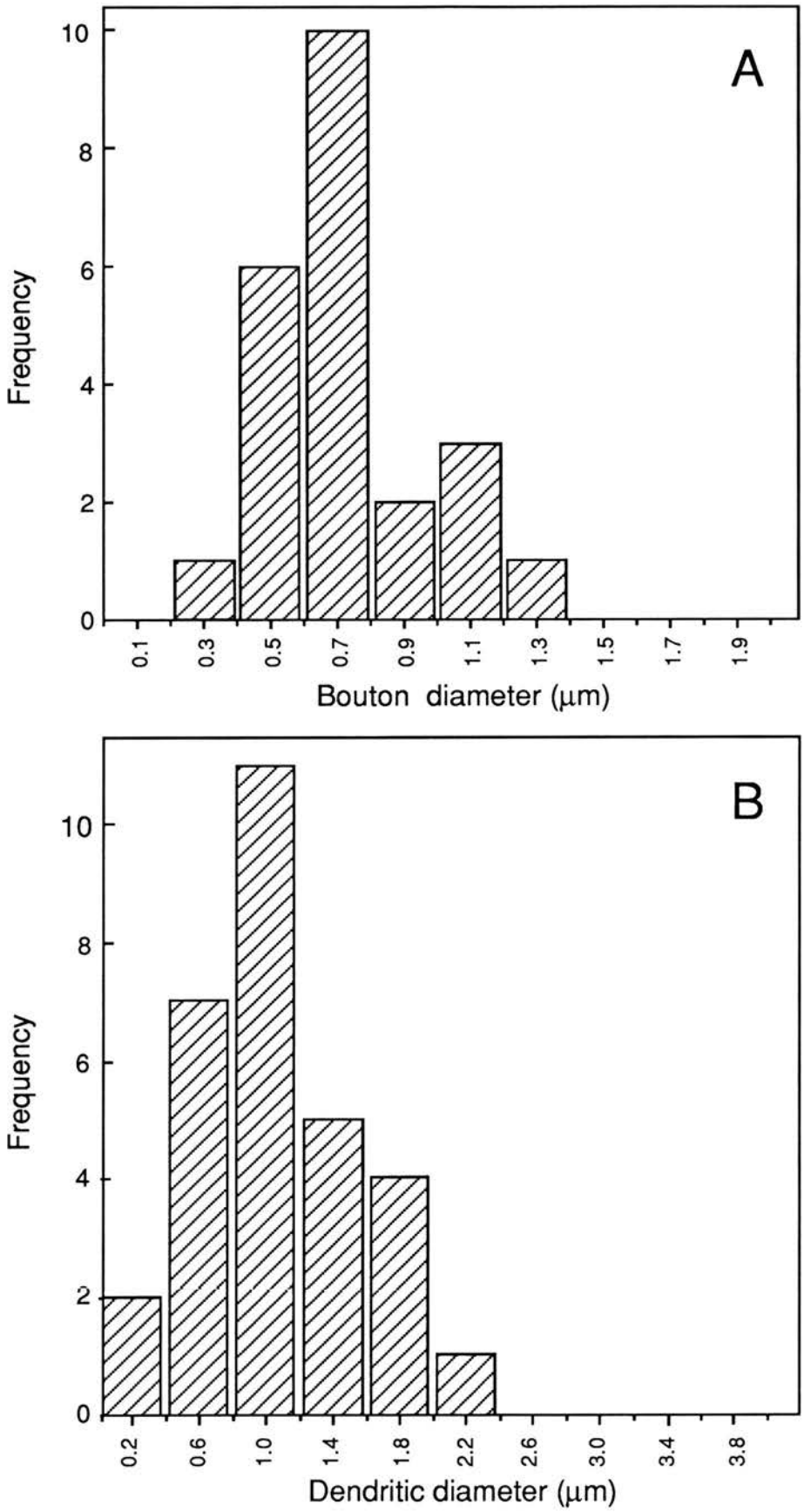

A
Frequency
0
2
4
6
8
10
0.1
0.3
0.5
0.7
0.9
1.1
1.3
1.5
1.7
1.9
Bouton diameter (μm)
B
Frequency
0
2
4
6
8
10
0.2
0.6
1.0
1.4
1.8
2.2
2.6
3.0
3.4
3.8
Dendritic diameter (μm)

Figure 30.

Light microscopic appearance of NPY-immunoreactivity throughout the feline spinal dorsal horn. The micrographs **A–F** were taken from transverse sections, whereas **G–J** were taken from sagittal sections. Large numbers of NPY-IR axons and varicosities are present in laminae I & II (**A**) (between the arrowheads), whereas the innervation to laminae III & IV is much sparser. The dorsomedial border of the dorsal columns (DC) is shown for orientation. In transverse sections (**B**) NPY terminals in laminae I & II occur as single, isolated punctate structures (arrowheads), whereas in sagittal sections (**G,H**) long, rostrocaudally orieted NPY fibres are also found. Moderate numbers of long, beaded NPY-IR axons are found in laminae III and IV (**C,D,J**), which often collect into bundles (**C**) (between the arrowheads). Occasional NPY fibres are present in laminae V and VI (**E,F**). NPY-IR terminals (arrowheads) were found in contact with neuronal cell bodies (*) in the superficial (**G**) and deeper (**E,I**) dorsal horn laminae. Scale bars: 0.25mm (**A**); 10μm (**E,F,H,I,J**); 20μm (**B,D,G**); 50μm (**C**).

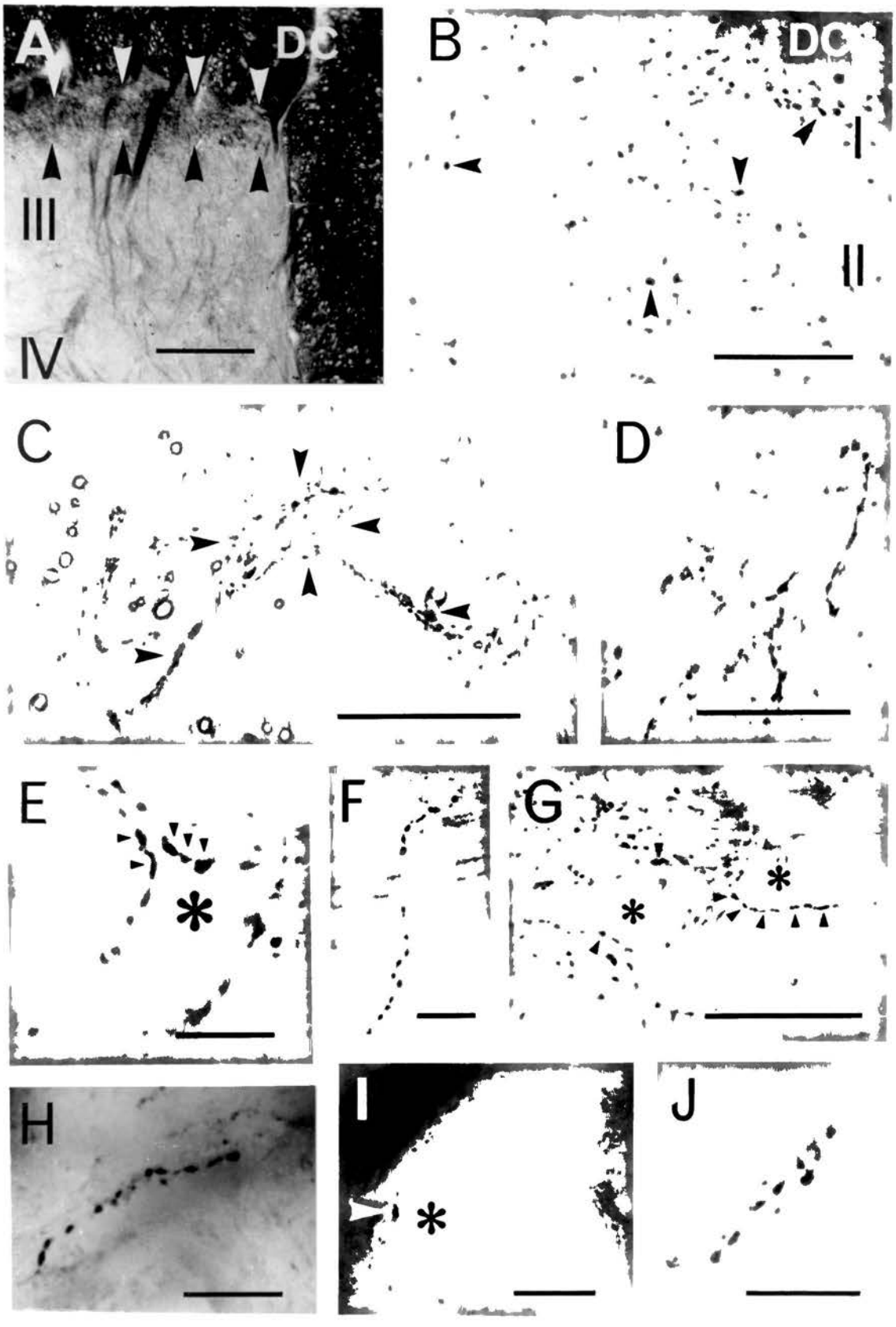

A
DC
III
IV
B
DC
I
II
C
D
E
F
G
H
I
J

Figure 31.

Electron micrographs of axo-dendritic synaptic junctions formed by NPY-IR boutons in laminae I and II. **A–C.** NPY-IR terminals form symmetric synaptic junctions (between the arrowheads) with small (**A**), medium (**B**) and large (**C**) dendrites. The synaptic junction formed by the NPY bouton in (**C**) is shown at higher magnification in the inset. These micrographs were taken from transverse sections. **E and F.** In sagittal sections, NPY-IR boutons commonly synapse (between the arrowheads) with rostro-caudally dendrites. The dendrite contacted by the NPY bouton in (**E**) is shown at lower magnification in (**D**).

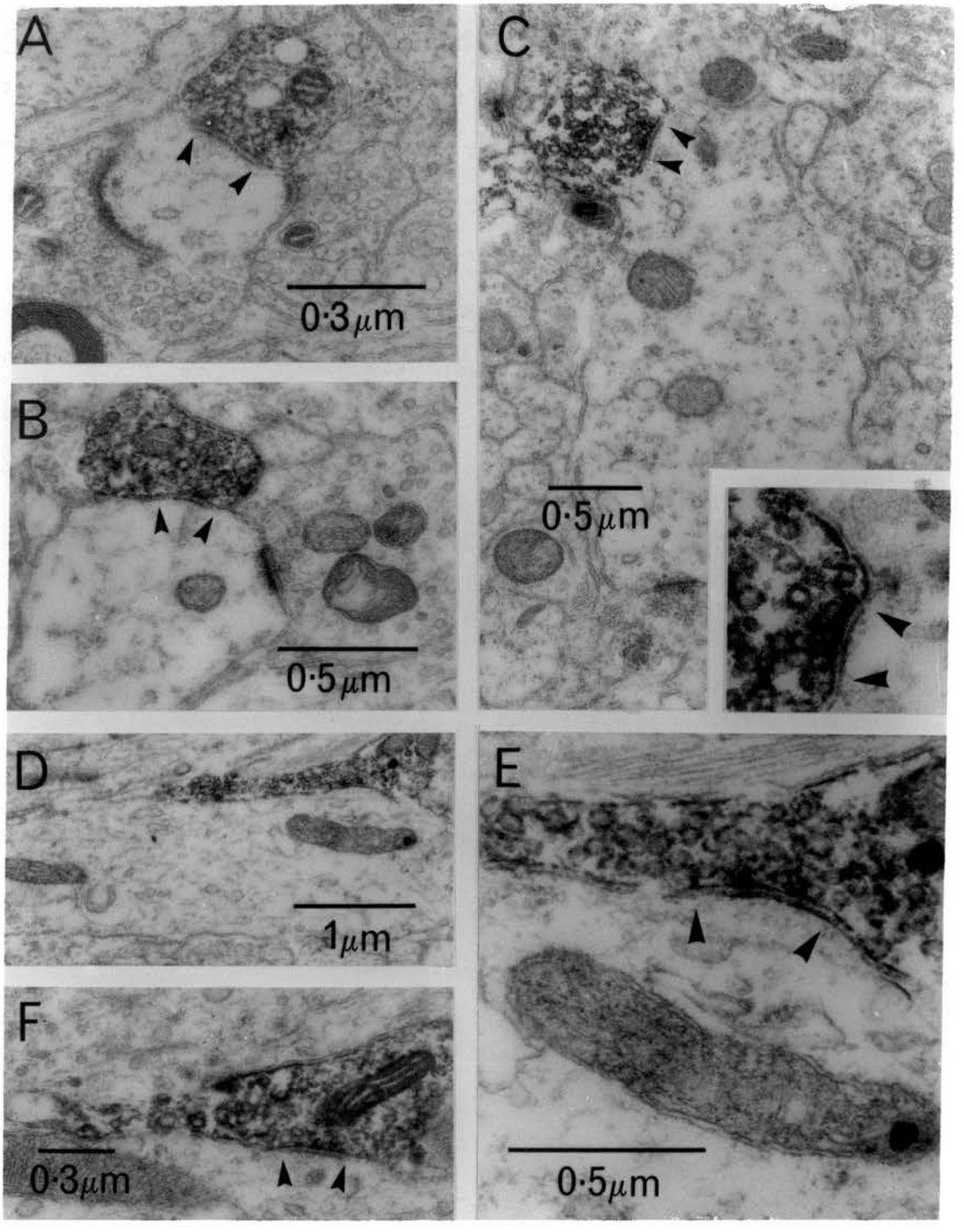

A
0·3μm
B
0·5μm
C
0·5μm
D
1μm
E
0·5μm
F
0·3μm

Figure 32.

Electron micrographs of axo-axonic synaptic junctions formed by NPY-IR boutons in lamina I. **A and B**. NPY-IR terminals form symmetric synaptic junctions (between the arrowheads) with small, unlabelled axon terminals (Ax). The insets show the synapses at higher magnification. Note the widening of the synaptic cleft, the symmetry of the membrane specializations and the accumulation of vesicles adjacent to the presynaptic membrane. **C and D**. NPY-IR terminals also synapsed upon central boutons in glomeruli. **C** shows the ultrastructural appearance of a central bouton (Cen), which is presynaptic to three unlabelled profiles (*) and is also apposed to an NPY-IR terminal (arrow). In a serial section (**D**), the NPY bouton forms a synaptic junction (between the arrowheads) with the central bouton. This is shown at higher magnification in the inset. The polarity of this synapse is from the NPY-IR terminal onto the central bouton.

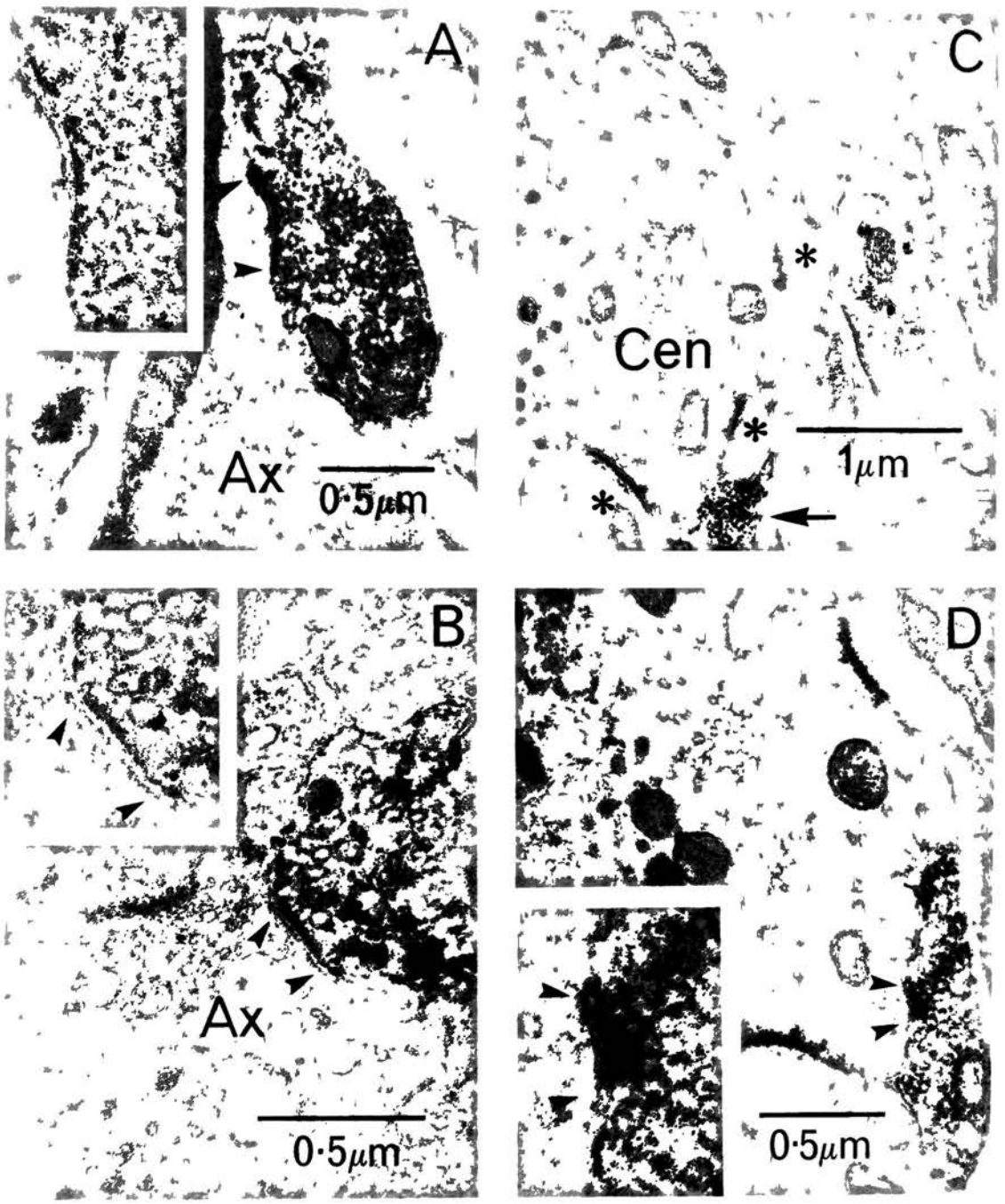

A
Ax
0·5μm
C
Cen
1μm
B
Ax
0·5μm
D
0·5μm

Figure 33.

Serial sections of an NPY-IR bouton from lamina II which forms an axo-axonic synaptic junction (between the arrowheads) with an unlabelled axon terminal (Ax). The polarity of this synapse is from the NPY-containing terminal onto the unlabelled axon. **A.** shows a section of the bouton which was taken mid-way through the synaptic junction. Note the obvious widening of the synaptic cleft and symmetry of the membrane specializations. **B.** shows the final section of the bouton in which the synaptic junction was present. Note the accumulation of vesicles adjacent to the presynaptic membrane and the marked postsynaptic thickening which gives the junction an asymmetric appearance in this particular section. This NPY-IR terminal also forms a synaptic junction with the dendrite D (arrow in Fig.B).

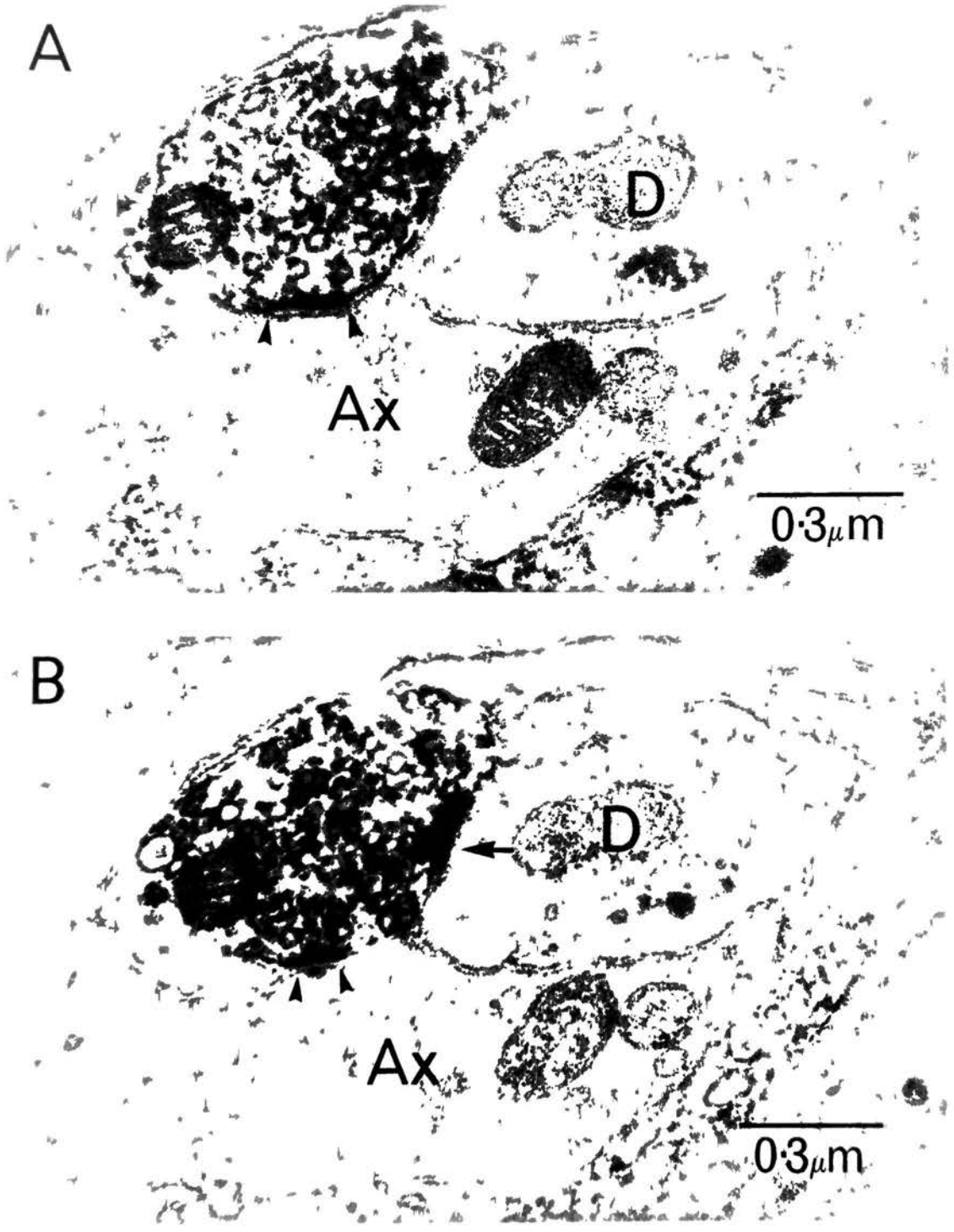

A
D
Ax
0·3μm
B
D
Ax
0·3μm

Figure 34.

A. shows an NPY-IR bouton which forms an asymmetric synaptic junction (between the arrowheads) with a large unlabelled axon terminal (Ax) in lamina II. In the serial section **B**, the synaptic junction (between the arrowheads) can be defined by the following criteria: (i) a well-defined synaptic cleft; (ii) a marked postsynaptic specialization producing an asymmetric synaptic junction; and (iii) an accumulation of vesicles adjacent to the presynaptic membrane. **C.** shows an NPY-containing bouton which is presynaptic to a central glomerular bouton which contains spherical synaptic vesicles of uniform diameter (RSV). The symmetric synaptic junction formed between these structures can be seen (between the arrowheads), but it is more clearly resolved at higher magnification (**D**).

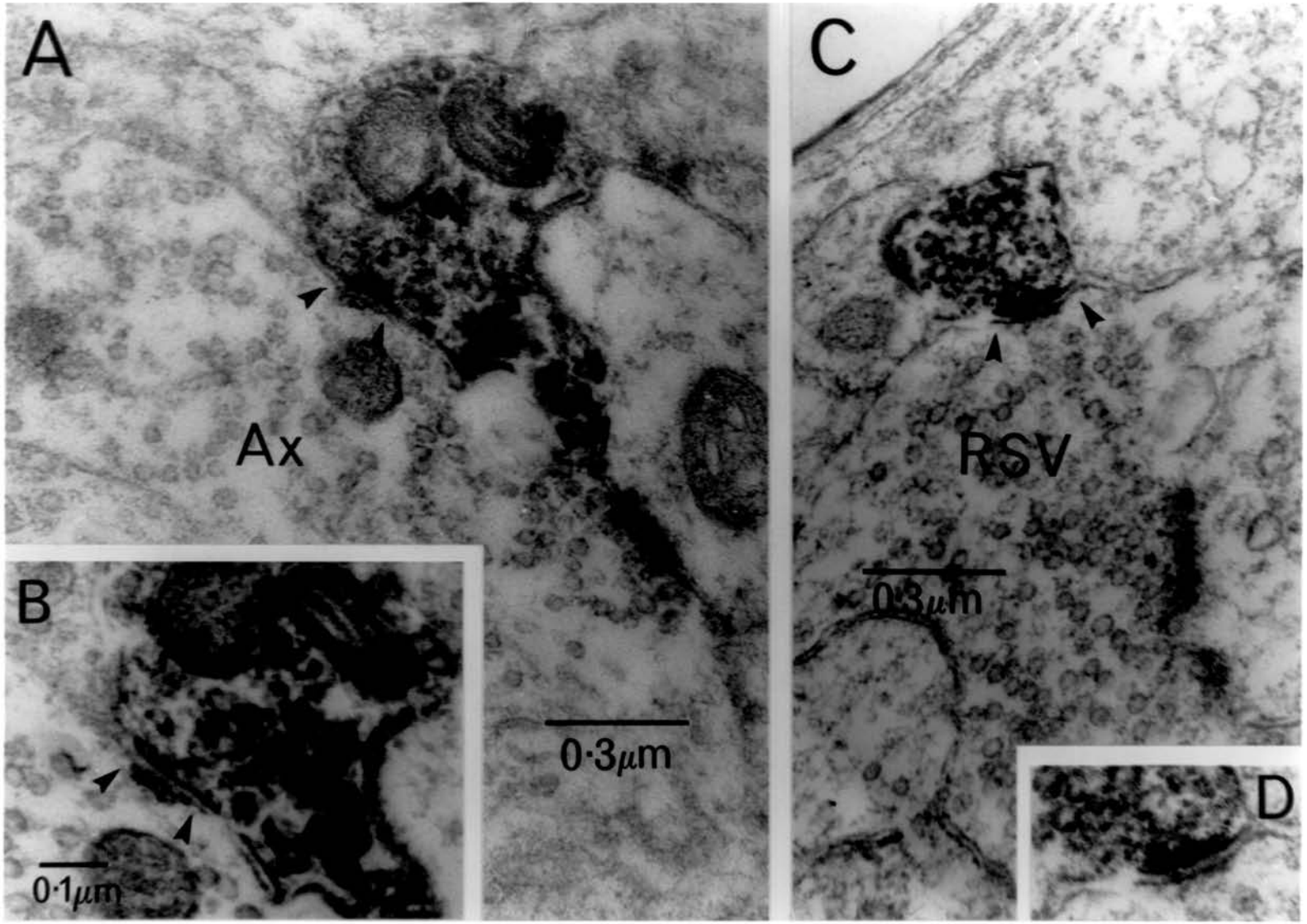

A
Ax
0·3μm
B
0·1μm
C
RSV
0·3μm
D

Figure 35.

Electron micrographs of NPY-IR boutons forming symmetric synaptic junctions with neurons in laminae I and II. (**A**). A small NPY-IR axon terminal in lamina I (arrow) is adjacent to a proximal dendrite (D) of a large neuronal perikaryon (P). Note the presence of the cell nucleus (N). At higher magnification (**B**), the synaptic junction can be seen (between the arrowheads). (**C**). A large NPY-IR terminal in lamina II (arrow) is apposed to a neuronal perikaryon (P). Note the presence of the nucleus (N). At higher magnification (**D**), a number of active zones can be seen (between the arrowheads).

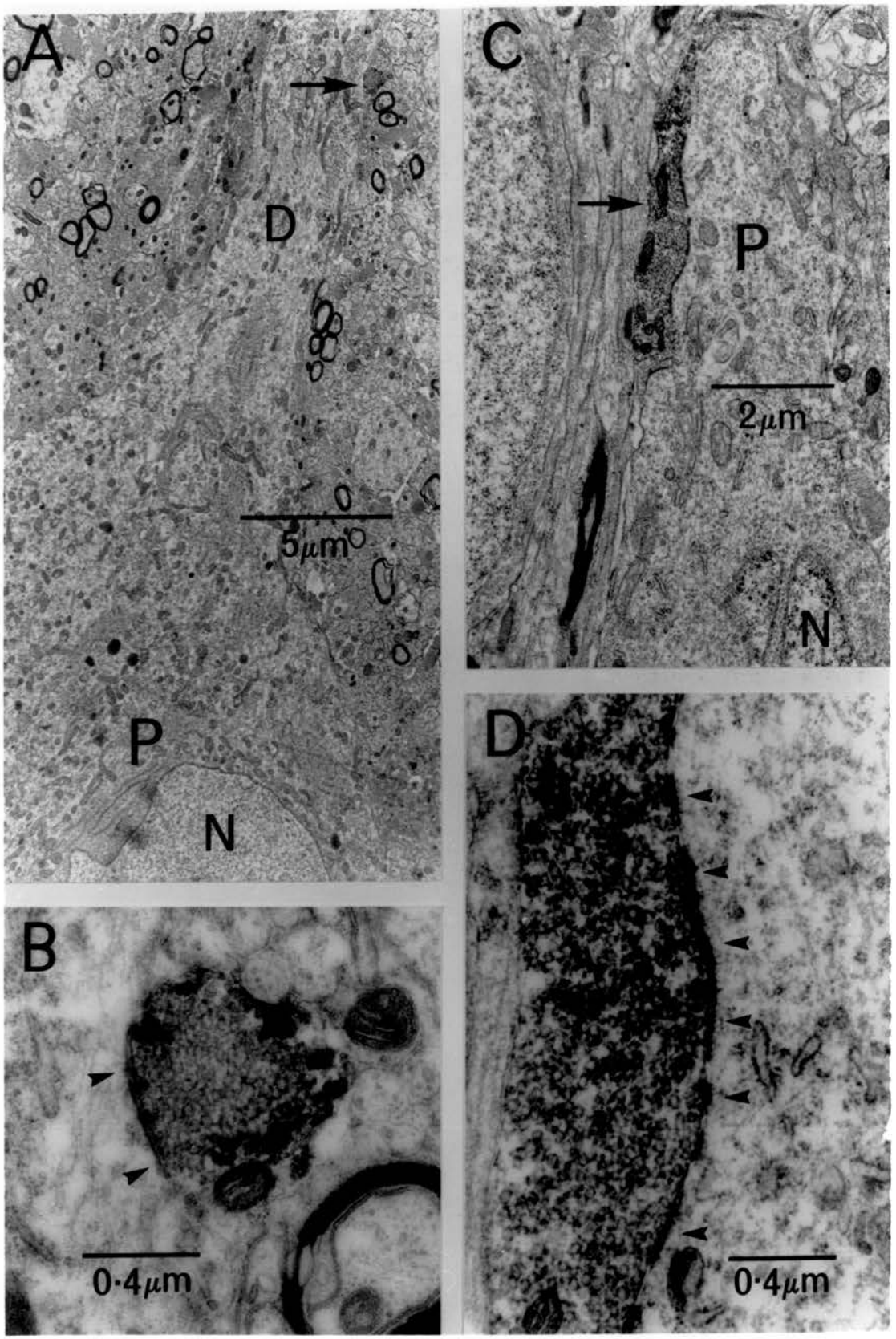

A
D
5μm
P
N
C
P
2μm
N
B
0·4μm
D
0·4μm

Figure 36.

Synaptic triad formed by a NPY-IR bouton in lamina II. (**A**). The NPY-IR bouton is presynaptic to a presumed axon terminal (Ax), and the synapse is shown between the arrowheads. Ax is itself presynaptic to a dendrite (D; arrow). In a serial section (**B**) the NPY-IR bouton is presynaptic to the same dendrite (between the arrowheads).

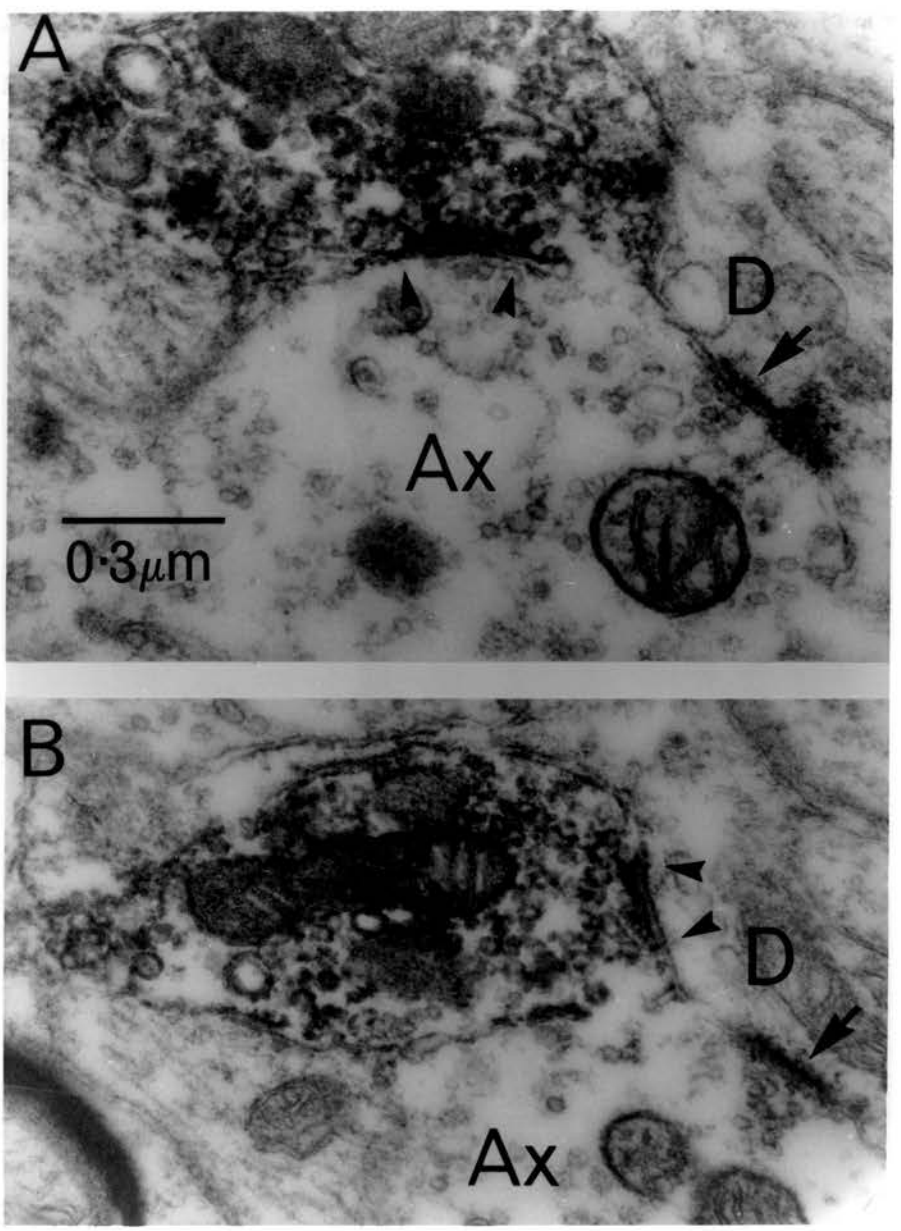

A
D
Ax
0·3μm
B
D
Ax

Figure 37.

Electron micrographs of the synaptic relationships formed by NPY-IR boutons in laminae III-VI of the dorsal horn. **A–D**. NPY-IR boutons form symmetric axo-dendritic synaptic junctions (between the arrowheads) with large (**A**), medium (**B,C**) and small (**D**) dendrites (D). **E–H**. NPY-IR boutons also form synaptic junctions (between the arrowheads) with unlabelled axon terminals (Ax). The axo-axonic synapse formed by the NPY bouton in (**E**) is shown at higher magnification in the inset.

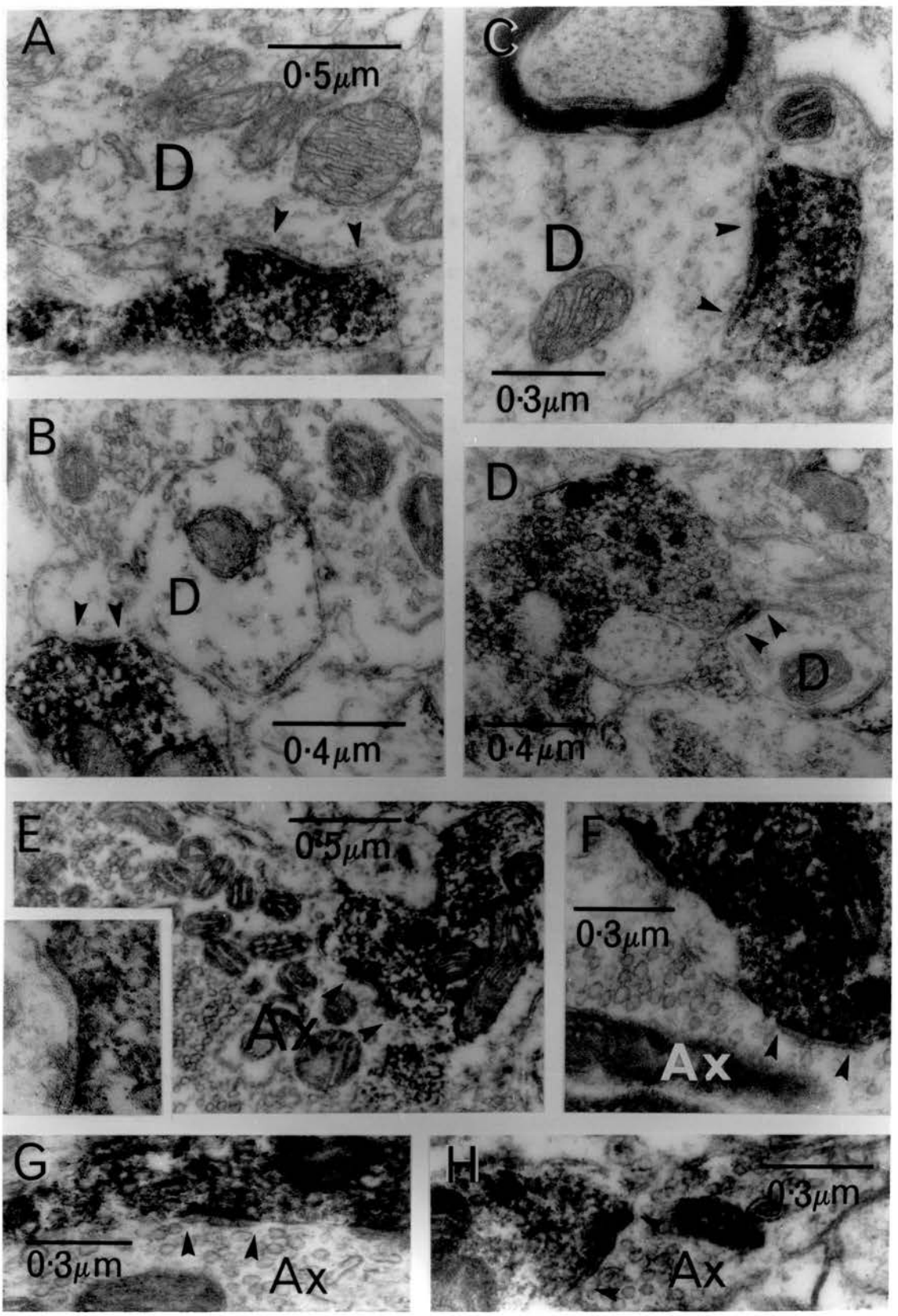

A
0·5μm
D
C
D
0·3μm
B
D
0·4μm
D
D
0·4μm
E
0·5μm
Ax
F
0·3μm
Ax
G
0·3μm
Ax
H
0·3μm
Ax

Figure 38.

(**A**). Electron micrograph of a vesicle-containing NPY-IR profile which formed a symmetric synaptic junction with an unlabelled dendrite (between the arrowheads). In a serial section (**B**), the postsynaptic dendrite is no longer present and the NPY-IR profile is apposed to a dense sinusoidal axon terminal (DSA). In a subsequent serial section (**C**), the NPY-IR profile is postsynaptic to the DSA axon. The asymmetric synaptic junction is shown between the arrowheads, and in the inset.

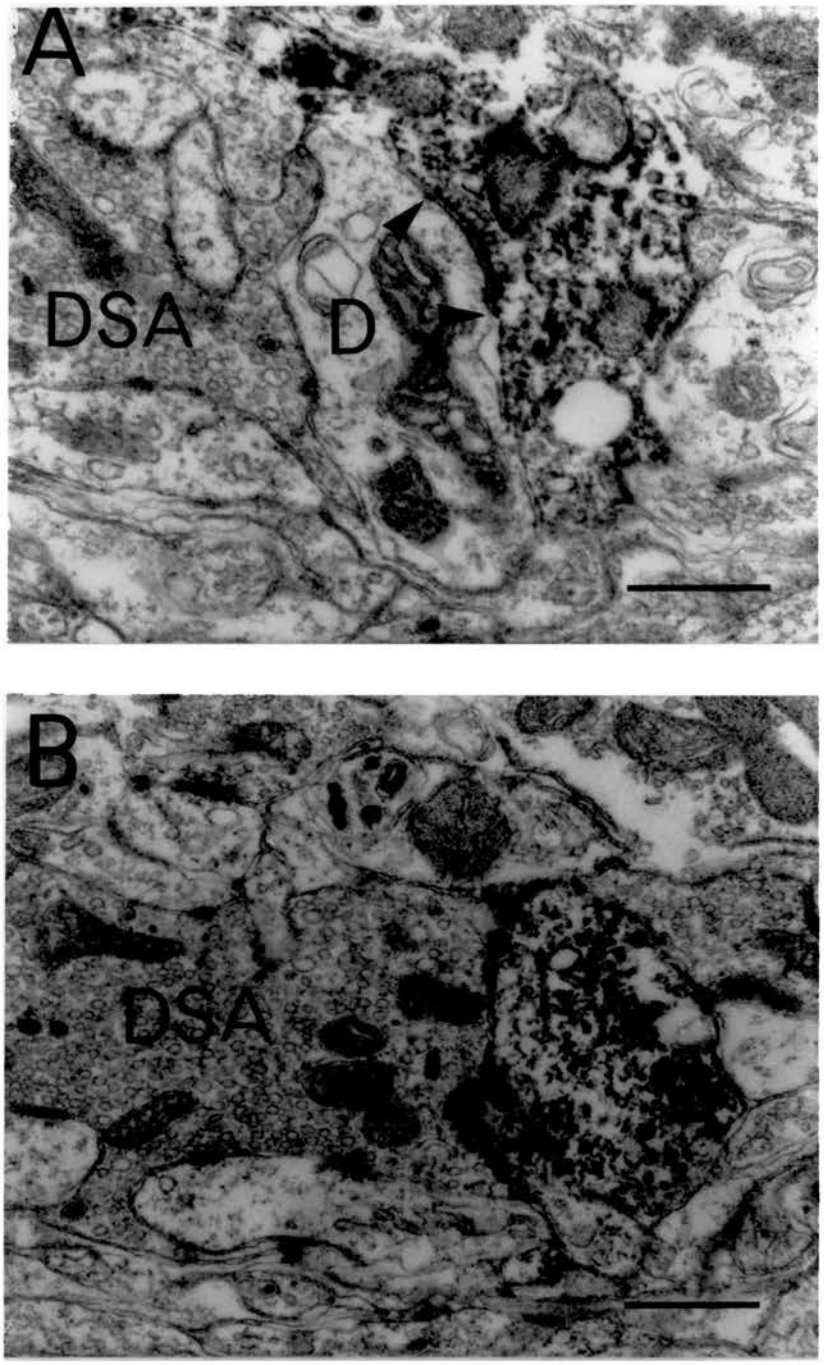

A
DSA
D
B
DSA
C
DSA

Figure 39.

(A). Frequency distribution histogram of NPY-IR bouton diameters. The majority (90%) of profiles fell within the range 0.5-1.2μm (mean diameter=0.75μm).

(B). Frequency distribution histogram of postsynaptic dendritic diameters. Immunolabelled boutons synapsed most commonly with medium-sized (0.5-2.0μm diameter) dendrites. Large dendrites (>2.0μm diameter) also received input from NPY-IR axonal endings, as did small (<0.5μm diameter) dendrites.

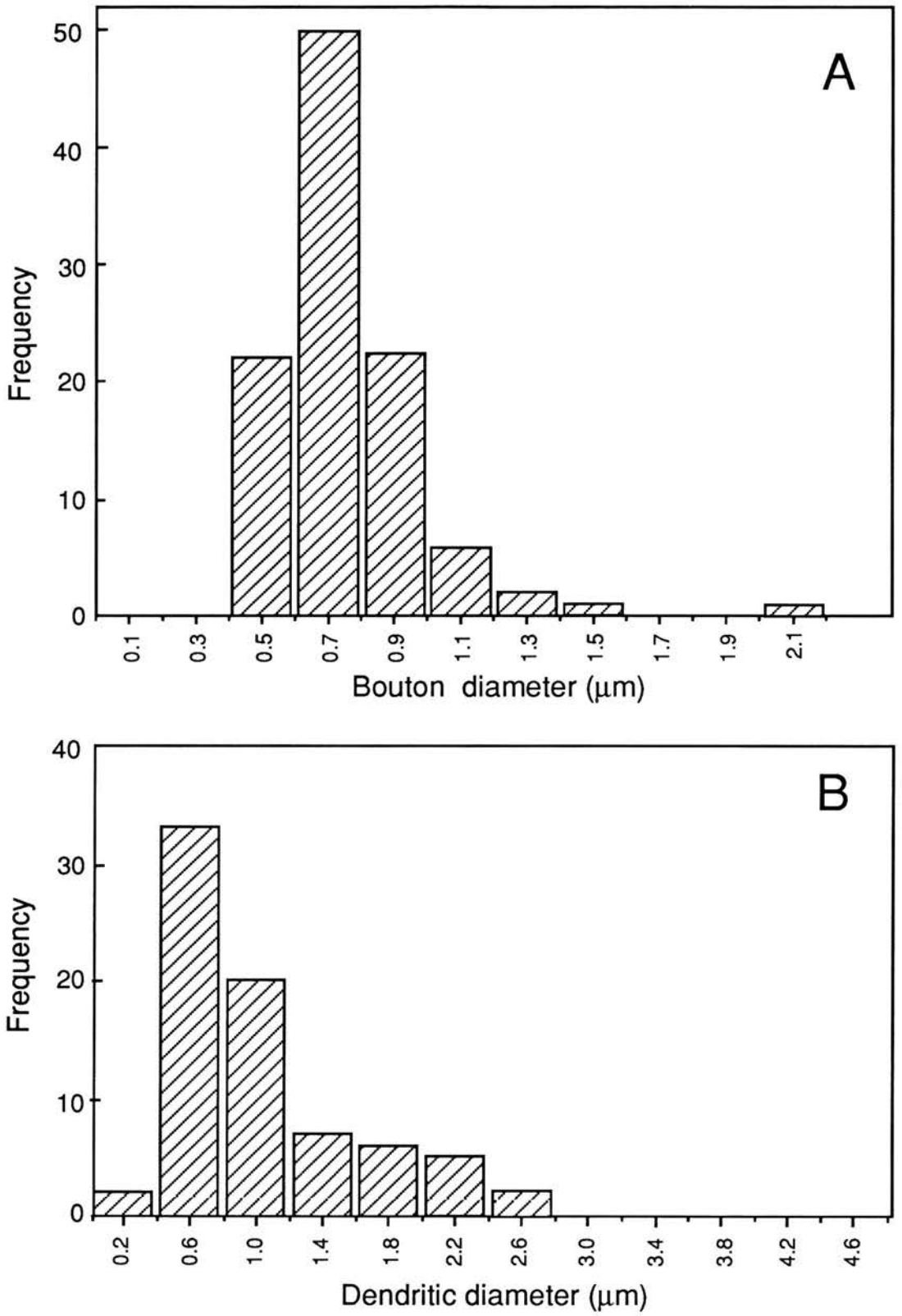

A
Frequency
50
40
30
20
10
0
0.1
0.3
0.5
0.7
0.9
1.1
1.3
1.5
1.7
1.9
2.1
Bouton diameter (μm)
B
Frequency
40
30
20
10
0
0.2
0.6
1.0
1.4
1.8
2.2
2.6
3.0
3.4
3.8
4.2
4.6
Dendritic diameter (μm)

Table 2.

Numbers of NPY-IR structures examined within each dorsal horn lamina and their postsynaptic targets.

Lamina	Number of Profiles Examined	Number forming Synaptic Junctions	Number of Axo-dendritic Contacts	Number of Axo-axonic Contacts	Number of Axo-somatic Contacts	Average Number of Synapses per Bouton
I	42	42	38	12	0	1.19
II	111	106	105	44	4	1.44
III	21	20	18	4	2	1.20
IV	20	18	14	5	0	1.06
V & VI	14	12	9	5	0	1.17
All	208	198	184	70	6	1.31

Discussion.

(1). ANALYSIS OF AXONS IMMUNOSTAINED FOR TYROSINE HYDROXYLASE (TH) OR DOPAMINE-ß-HYDROXYLASE (DBH).

(a). **Distribution and orientation of fibres.**

Axons immunoreactive for TH and DBH were present throughout the spinal dorsal horn, with laminae I,II and IV having the densest innervation.

Within the superficial layers (laminae I and II) the axons exhibit a prominant rostrocaudal orientation, with the highly varicose fibres travelling for considerable distances without branching. This pattern of descent for catecholamine (CA)-containing fibres in these layers has been described previously (Carlsson et al., 1964; Dahlström and Fuxe, 1965; Fritschy and Grzanna, 1990). The dendritic arbors of neurons are oriented in a similar way (Gobel, 1978; Gobel et al., 1980; Ramón Y Cajal, 1909), and it seems reasonable to suggest that the TH- and DBH-immunoreactive fibres form synaptic contacts with these parallel-arranged dendrites.

Sugiura et al. (1986) have shown using a *Phaseolus vulgaris* leucoagglutinin intracellular staining technique in the guinea-pig that cutaneous unmyelinated (C) fibres within laminae I and II distribute as long, rostro-caudally oriented strands, with relatively few branches. Similarly, Gobel et al. (1981) observed that ultrafine varicose axons within lamina I, labelled following application of horseradish peroxidase (HRP) to cut dorsal

roots, travel for considerable distances in the rostrocaudal direction without branching. The similar orientation of CA-containing axons and fine primary afferent axons in laminae I and II could imply a close association between the the two groups. However, the ultrastructural observations (see below) did not reveal immunolabelled boutons synapsing with other axon terminals.

Within laminae III and IV, the orientation of the immunolabelled axons changes from rostrocaudal to dorsoventral. Many laminae III and IV cells possess dorsally directed dendrites, which extend upwards often penetrating laminae I-III (Brown, 1981; Brown et al., 1977b; Brown and Fyffe, 1981; Schiebel and Schiebel, 1968; Szentágothai, 1964). The orientation of the immunolabelled axons therefore appears to change to accomodate the different arborization pattern formed by the cells in this region. In this way, the CA-containing varicose fibres may remain parallel to their target dendrites.

TH- and DBH-immunoreactive fibres were not uniformly distributed across the mediolateral extent of the dorsal horn. In a similar way to the distribution of corticospinal axons (Cheema et al., 1984), the descending CA-containing fibres were predominantly located in the medial part of the dorsal horn. A heterogenous arrangement of the dorsal horn in the mediolateral direction has also been reported with respect to certain

primary afferent inputs (Molander and Grant, 1985; Swett and Woolf, 1985). These authors mapped the distribution of hind-limb nerves in the rat and found that the tibial nerve that innervates the glabrous skin on the hind-foot projects to the medial third of the horn. Furthermore, Réthelyi et al. (1986) have observed differences in axonal sprouting between the medial and lateral sectors following neonatal capsaicin treatment.

(b). **Ultrastructural observations.**

Observations with the electron microscope have shown that descending catecholaminergic projections to the cat lumbosacral spinal dorsal horn form symmetric (Gray type II) synaptic specializations with their postsynaptic targets. The majority of immunolabelled boutons (88%) was found in association with dendrites, while a few (6%) were seen in contact with neuronal somata. On no occasion were axo-axonic synapses observed. It cannot be stated conclusively that CA-containing boutons do not contact other axon terminals, but if this type of synaptic arrangement does exist in the cat, then it is very rare. The majority of axo-dendritic synapses was found on medium-sized dendrites but contacts upon large calibre dendritic shafts and small diameter dendrites were not uncommon. It appears, therefore, that contacts are found throughout the dendritic arbors of dorsal horn cells. A few boutons were found which did not form synapses (6%).

These findings are in contrast to those described by Descarries et al. (1977) and Seguéla et al. (1990) for

ascending catecholaminergic systems in the rat cortex. These authors found that the majority of CA-containing terminals (80-95%) lacked the membrane specializations of typical synaptic contacts. In this respect, it was proposed that the ascending projections exert a diffuse effect on postsynaptic structures.

Prior to, during and subsequent to the publication of these results, a number of abstracts and papers have been published which describe the organization of spinal CA-containing systems in the rat. However, findings in this species are equivocal. Studies using autoradiography (Ruda et al., 1979) and DBH-immunocytochemistry (Akeyson et al., 1983; Hagihira et al., 1990) have found that the majority of noradrenergic terminals in the rat dorsal horn form typical synaptic contacts. These were mostly (Akeyson et al., 1983) or exclusively (Hagihira et al., 1990; Ruda et al., 1979) asymmetric (Gray type I). The synaptic incidence of contacts formed by dopamine-positive boutons in the rat dorsal horn has been described by Ridet et al. (1992). In cervical segments, dopaminergic terminals had a relatively low synaptic incidence (34%), but at thoraco-lumbar levels the incidence was high (76%). These observations are in contradiction with those reported by Rajaofetra et al. (1992) and Ridet et al. (1993) using a specific antiserum against NA. For these authors, the majority of noradrenergic varicosities observed in the rat dorsal horn (71-75%) presented non-synaptic contacts, and they

proposed that the spinal NA system may, like that of the cortex, act in a diffuse manner (volume transmission). The disparity between these results in the rat (Rajaofetra et al., 1992; Ridet et al., 1993), and results presented in this thesis for the cat and by other authors for the rat (Akeyson et al., 1983; Hagihira et al., 1990; Ruda et al., 1979) probably represent species and sub-species differences.

The postsynaptic targets of CA-containing boutons in the rat (Akeyson et al., 1983; Hagihira et al., 1990; Rajaofetra et al., 1992; Ridet et al., 1992; Ruda et al., 1979) are the same as those described in the present study for the cat, although Ridet et al. (1993) have suggested that a small number of NA-containing terminals in the rat form axo-axonic synapses.

Boutons immunolabelled for TH and DBH were found to contain two distinct types of vesicle: (i) small, irregularly-shaped agranular vesicles, and (ii) prominant dense-core vesicles. The small agranular vesicles are likely to contain CA's since they are rendered electron dense by permanganate fixation (Satoh et al., 1979). This type of precipitation reaction is selective for monoaminergic neurotransmitters (Hökfelt and Jonsson, 1968; Hökfelt and Ljungdahl, 1972). Dense-core vesicles can be demonstrated in axon terminals containing transmitters other than CA's (Hökfelt and Ljungdahl, 1972). Recent evidence indicates that some of these vesicles contain neuropeptides (Merighi et al., 1989; Riberio da Silva et al., 1989) and a number of studies

have demonstrated the co-existence of CA's with neuropeptides in the central nervous system. For instance, 23% of CA-containing cells in the locus coeruleus of the rat have been shown to be immunoreactive for neuropeptide Y (Everitt et al., 1984; Holets et al., 1988; Yamazoe et al., 1985) and, in addition, the majority of A6 profiles (82%) were also positive for galanin (Holets et al., 1988; Melander et al., 1986) and enkephalin (Charnay et al., 1982). Therefore, the dense core vesicles observed within TH- and DBH-immuno-reactive boutons may contain some, or all, of these peptides. Results from the present studies show that the dense core vesicles contain DBH but not TH.

Functional considerations.

A number of groups have investigated the actions of adrenergic agonists upon dorsal horn neurons which were activated by peripheral stimulation. In general, the predominant effect is an inhibition of activity (Belcher et al., 1978; Davies and Quinlan, 1985; Engberg and Ryall, 1966; Fleetwood-Walker et al., 1985, 1988; Headley et al., 1978; Howe and Zieglgänsberger, 1987; Satoh et al., 1979), although occasional excitation is also seen (Belcher et al., 1978; Howe and Zieglgänsberger, 1987; Millar et al., 1993; Satoh et al., 1979). These findings are consistent with the effects of electrically stimulating brainstem nuclei known to contain catecholaminergic cell bodies (Fleetwood-Walker et al.,

1988; Girardot et al., 1987; Hodge et al., 1983; Liu and Zhao, 1992; Mokha et al., 1985; Sasa et al., 1974; Zhao and Duggan, 1988).

On the basis of results from electrophysiological and biochemical studies, it appears that CA's exert both presynaptic (Carstens et al., 1987; Carstens and Zimmermann, 1981; Jeftinija et al., 1981; Kamisaki et al., 1993; Kuraishi et al., 1985a; Takano et al., 1993) and postsynaptic (Belcher et al., 1978; Carlton et al., 1991; Engberg and Ryall, 1966; Hylden & Wilcox, 1983; Millar et al., 1993; North and Yoshimura, 1984) actions in regulating sensory transmission through the dorsal horn. In the present study, no immunolabelled boutons were found forming synaptic junctions with other axon profiles, and it is concluded that in the cat catecholaminergic axons do not directly act upon primary afferent terminals to produce presynaptic inhibition.

Ultrastructural analysis confirms that TH- and DBH-immunoreactive boutons form synaptic contacts with the dendrites and somata of cells throughout the dorsal horn. These observations favour the idea of a postsynaptic mechanism of action for catecholaminergic axons within the spinal dorsal horn. Furthermore, the distribution of CA-containing boutons in the spinal dorsal horn suggests that CA's may regulate the responses of a number of different neuronal cell types. Lamina I contains projection neurons of the spinothalamic tract (Carstens and Trevino, 1978) in addition to local circuit neurons (Bennett et al., 1981). The high density of TH- and DBH-

containing fibres in this region could depress activity of thalamic projection neurons as well as inhibit local circuit neurons. Lamina II of the dorsal horn is composed almost entirely of local circuit neurons. The two major types of cell are the stalked cell and the islet cell (Gobel, 1978; Gobel et al., 1980), each of which could receive contacts from catecholaminergic terminals. The neuropil of the superficial dorsal horn also contains dendrites of neurons with cell bodies in deeper laminae of the dorsal horn (Bennett et al., 1984; Brown and Fyffe, 1981). Therefore, it is possible that distal dendrites of these cells receive catecholaminergic input via laminae I and II. Laminae III-V contains projection neurons of the spinocervical (Brown et al., 1977b, 1980a; Enevoldson and Gordon, 1989b) and post-synaptic dorsal column system (Bennett et al., 1983, 1984; Brown & Fyffe, 1981; Enevoldson and Gordon, 1989a; Giesler et al., 1984; Rustioni, 1977; Rustioni and Kaufman, 1977). An inhibitory action by CA's upon these cells would modify spinal input to the lateral cervical nucleus and the dorsal column nuclei.

A number of authors have reported that CA's, when applied ionophoretically to the dorsal horn, depress nociceptive responses with greater efficacy than responses to innocuous stimuli (Belcher et al., 1978; Davies and Quinlan, 1985; Fleetwood-Walker et al., 1985, 1988; Headley et al., 1978; Millar et al., 1993) and it has been suggested that the descending catecholaminergic

neurons selectively depress activity generated by noxious stimuli. Other authors, however, have failed to demonstrate any selectivity (Howe and Zieglgänsberger, 1987; Satoh et al., 1979). The postsynaptic target neurons of TH- and DBH-labelled boutons were not identified in this part of the study. However, on the basis of the primary afferent inputs to the different laminae of the dorsal horn, several inferences may be drawn. The high density of CA-containing axons within laminae I and II, where high threshold cutaneous Aδ and C fibres terminate (Light and Perl, 1979; Sugiura et al., 1986), infers that a depression of nociceptive transmission may occur in this region. Furthermore, in laminae III and IV, where Aαß fibres terminate (Brown, 1981), the catecholaminergic axons may depress transmission of low threshold inputs. Until the precise nature of the postsynaptic targets of catecholaminergic axons is resolved, interpreting the effects observed during CA ionophoresis will remain difficult. This would include knowing whether CA-containing axo-dendritic synapses on distal dendrites are adjacent to excitatory synapses conveying nociceptive information. A selective inhibition of nociceptive pathways may be achieved by such a local circuit through shunting inhibition.

Brainstem stimulation studies indicate that a selective inhibition of nociceptive pathways can be achieved through activation of the locus coeruleus (Hodge et al., 1983; Liu and Zhao, 1992; Mokha et al., 1985; Zhao and Duggan, 1988) the A1-C1 group (Liu and Zhao,

1992) and the dopaminergic A11 cell group (Fleetwood-Walker et al., 1988). The subcoeruleus-parabachial nuclei (Girardot et al., 1987) and the Kölliker-Fuse nucleus (Zhao and Duggan, 1988) (A7 group) produce a non-selective inhibition of sensory transmission. An intriguing finding to emerge from these studies was the insensitivity of coerulospinal inhibition to reversal by adrenergic antagonists. Hodge et al. (1983) found that inhibition from the locus coeruleus was unaltered by depletion of brain and spinal cord NA with reserpine (1-2mg/kg,i.p). Furthermore, Zhao and Duggan (1988) found that while idazoxan antagonized NA-induced inhibition, it failed to block the inhibition of dorsal horn neurons induced by stimulation of the locus coeruleus or Kölliker-Fuse nucleus. One possible explanation of these results is that other transmitters are co-released with NA, and that depletion of NA alone is insufficient to reduce a postsynaptic action. It has been discussed earlier that the dense-core vesicles observed within TH- and DBH-positive boutons are likely storage sites for candidate transmitter substances such as enkephalin, galanin and neuropeptide Y. It is significant, therefore, that a co-release of NA with neuropeptide has been reported in the peripheral nervous system (Stjarne and Lundberg, 1986), and neuropeptide Y and galanin have been shown to depress nociceptive reflexes in rats (Hua et al., 1991; Yanagisawa et al., 1986).

(2). IDENTIFICATION OF SOME OF THE SPINAL NEURONS WHICH ARE POSTSYNAPTIC TO CATECHOLAMINERGIC AXONS.

The next stage of the study was to identify some of the spinal neurons which receive a direct input from CA-containing axon terminals.

Laminae III and IV of the dorsal horn contain, amongst other things, large numbers of CA-containing axonal varicosities in addition to the perikarya and dendritic arbors of postsynaptic dorsal column (PSDC) & spinocervical tract (SCT) neurons (Bennett et al., 1983, 1984; Brown et al., 1977b, 1980a, 1983; Brown and Fyffe, 1981; Enevoldson and Gordon, 1989a,b; Giesler and Cliffer, 1985; Giesler et al., 1984; Rustioni, 1973, 1974, 1977; Rustioni and Kaufman, 1977). Hence, there is opportunity for interaction between CA-containing axons and these two groups of projection neurons. Furthermore, NA can alter the cutaneous response characteristics of PSDC and SCT cells when ionophoresed into the dorsal horn (Fleetwood-Walker et al., 1985). It therefore follows that PSDC and SCT neurons might be projection targets of descending CA-containing axons. Hence, these groups of dorsal horn neurons were retrogradely labelled by means of a HRP pellet implantation technique (Enevoldson et al., 1984; Enevoldson and Gordon, 1989a,b) to test this hypothesis.

(A). POSTSYNAPTIC DORSAL COLUMN (PSDC) NEURONS.

(a). **Identification of PSDC neurons.**

The electrophysiological studies of Angaut-Petit (1975a) have shown that 87% of ascending second-order axons in the thoracic dorsal columns project to the first cervical segment. At C2-C3, it is probable that all dorsal column axons originating in the lumbar cord belong to this pathway.

Anatomical studies in the cat have shown differences in the laminar distribution of labelled cells following HRP applications to the thoracic dorsal columns and the dorsal column nuclei (DCN). Rustioni and Kaufman (1977) and Enevoldson and Gordon (1989a) found that lumbar retrograde labelling was almost entirely confined to laminae III-V after HRP was applied to the DCN. The cells labelled by Rustioni and Kaufman (1977) also included the small population of spino-DCN cells ascending the DLF (Enevoldson and Gordon, 1989b), since these authors made no lesion in the DLF. When HRP was placed into the dorsal columns at T9-T12, an additional cluster of cells was revealed in medial laminae VI-VII, as well as a few cells in lamina I (Bennett et al., 1983). I also observed this pattern of retrograde labelling, and concluded that the cells labelled in laminae I, VI and VII are long propiospinal neurons and correspond to the small population (13%) of non-PSDC, second-order cells observed ascending the thoracic dorsal columns by Angaut-

Petit (1975a). On this basis, only labelled cells within laminae III-V were examined, since these neurons probably project to the DCN. In this context, it may be significant that Rustioni and his colleagues only found retrograde labelling within laminae I and VI-VII when their medullary HRP injections were not restricted to the DCN (Rustioni, 1976; Rustioni and Kaufman, 1977). However, despite these precautions, one cannot eliminate the possibility that a few of the cells examined may have been propiospinal.

The agar-HRP implantation technique employed in this study depends upon uptake of HRP by the severed ends of axons but not by axons of passage. Considerable evidence supporting this claim has been presented in a number of publications. Enevoldson and Gordon (1989a) and Bennett et al. (1983) performed control experiments involving lesions of the DLF caudal to the site of the implant. Both studies revealed that there was no alteration in retrograde labelling as a result of the additional lesion and concluded that HRP had not been taken up by axons in the DLF. In addition, Enevoldson et al. (1984) found a critical relationship between the number of cells labelled and the extent of the dorsal column lesion. Examination of the lesion sites in these experiments indicated that there was some spread of HRP into the DLF. However, as no damage of the DLF was observed in any of the sections surrounding the implant site, it is unlikely that any labelling of axons in this tract occurred.

It is also possible that spread of HRP into the grey

matter surrounding the lesion site could result in labelling of propiospinal neurons with axons terminating in this region. This is unlikely, however, since Bennett et al. (1983) found that lesioning the dorsal columns caudal to the implant resulted in a total absence of retrograde labelling.

(b). **Innervation of neurons by catecholaminergic axons.**

Combined light and electron microscopic analysis has shown that lumbosacral PSDC neurons in the cat are innervated by CA-containing axons originating in the brain. Light microscopic observations suggest that about 60% of cells possess CA-containing terminals closely apposed to their somata and/or proximal dendrites, with some cells receiving up to 22 contacts. However, estimates of frequency of contacts are likely to be limited in this study for several reasons. (i). Ultrastructural studies presented earlier in this thesis indicated that the majority of synaptic contacts formed by CA-containing boutons are on fairly small (less than 2μm diameter) dendritic shafts, which are probably distal dendrites. As the retrograde labelling technique employed in this study resulted in the labelling of somata and proximal dendrites only, it is probable that the majority of contacts between CA-containing fibres and PSDC neurons would not be detected. A greater frequency of contact might be expected if PSDC neurons were intracellularly stained with HRP, since this technique

produces a much more extensive labelling of the dendritic arbor, including the distal dendrites. Alternatively, a more sensitive retrograde label, such as lectin-conjugated HRP or cholera toxin subunit b, could have been used in conjunction with cobalt intensification. (ii). The presence of glutaraldehyde in the fixative reduces immunocytochemical staining by denaturing the target antigen. Hence it is unlikely that all of the catecholaminergic innervation to the dorsal horn was actually labelled. In addition, the high concentrations of paraformaldehyde in the fixative, along with using a relatively insensitive chromogen (DAB), would also reduce the number of labelled cells.

Although light microscopy revealed that over 60% of PSDC neurons receive contacts from CA-containing axons, its resolution (0.2μm) is not sufficient to exclude the possibility that structures in apparent apposition are actually separated by an intervening neuronal or glial process, or that a varicosity in contact with a labelled cell is actually synapsing on some adjacent unlabelled structure and not the cell itself. Correlated ultrastructural analysis, however, verified that many contacts between CA-containing fibres and labelled neurons (12/20) were regions of synaptic association. These findings suggest that many of the contacts seen with the light microscope alone were regions of synaptic interaction. However, 8 axonal swellings which appeared to contact labelled cells were found not to form synapses with the cells when viewed ultrastructurally and

therefore it cannot be assumed that all contacts observed with the light microscope are synaptic connections. Seven of these boutons synapsed on an adjacent, unlabelled dendrite and one bouton did not form a synaptic junction.

(c). **Functional considerations.**

Previous functional studies have shown that the spinal catecholaminergic system plays an important role in controlling the transmission of nociceptive information to supraspinal sites. For instance, in behavioural studies the intrathecal administration of CA's inhibits reflexes evoked by noxious cutaneous stimuli (Jensen and Smith, 1983; Jensen and Yaksh, 1984; Jensen et al., 1984; Kuraishi et al., 1979b, 1985b; Liu et al., 1992; Reddy and Yaksh, 1980; Tjolsen et al., 1990; Yaksh and Reddy, 1981). A similar analgesia results from stimulation of the locus coeruleus (Jones and Gebhart, 1986; Segal and Sandberg, 1977), the A7 group (Yoemans et al., 1992), and the A5 group (Burnett and Gebhart, 1991; Millar and Proudfit, 1990).

In addition, electrophysiological recordings from unidentified dorsal horn neurons (Belcher et al., 1978; Davies and Quinlan,'85; Millar et al., 1993), spino-cervical tract neurons (Fleetwood-Walker et al., 1985, 1988), PSDC neurons (Fleetwood-Walker et al., 1985) and neurons of the spinothalamic and spinomesencephalic pathways (Fleetwood-Walker et al., 1988) have

demonstrated that excitation induced by noxious cutaneous stimulation may be inhibited by CA's. In these studies nociceptive responses were depressed to a much greater extent than responses to innocuous stimuli, and the authors proposed that the descending CA pathways act relatively selectively to inhibit nociceptive transmission. On this basis, it has been suggested (Fleetwood-Walker et al., 1985) that the inhibitory action of CA's occurs at a site remote from the cell being recorded from, possibly upon the terminals of nociceptive primary afferents, to inhibit transmitter release. However, ultrastructural studies described earlier have shown that CA-containing terminals synapse upon dendrites and somata only. Furthermore, other groups have found that noradrenaline attenuates the responses to noxious and non-noxious stimuli with equal potency (Howe and Zieglgänsberger, 1987; Satoh et al., 1979). Hence, the precise effects of CA's in the spinal cord are at present uncertain. In this part of the study it was shown that spinal neurons which project to the dorsal column nuclei via the dorsal columns (PSDC neurons) are innervated by CA-containing axons. Electrophysiological studies have demonstrated that most of these neurons (62-77%) respond to a wide range of stimuli including hair movement, noxious pinch and heat, and only a very small minority are nociceptive specific (about 3%) (Angaut-Petit, 1975b; Brown et al., 1983; Giesler and Cliffer, 1985). Hence, the predicted effect of the catecholaminergic axons innervating almost all PSDC

neurons would be to suppress excitation generated by both light-tactile and nociceptive cutaneous stimuli.

Headley et al. (1978) recorded from multireceptive neurons in laminae IV and V and compared the effect of ionophoresing NA in the substantia gelatinosa with ejection in the vicinity of the neuron being tested. Although ionophoresis in the substantia gelatinosa produced a specific inhibition of nociception, ionophoresis close to the cell body resulted in a much less selective action. This non-specific inhibition by NA of laminae IV-V neurons is therefore compatible with the suggestion that the direct action of CA-containing axons on PSDC neurons would be a general suppression of excitation and not selective inhibition of nociceptive input. Selective inhibition of noxious input to lamina IV cells, observed when NA is ionophoresed into the substantia gelatinosa, might be due to hyperpolarization of lamina II interneurons (North and Yoshimura, 1984) involved in nociceptive polysynaptic pathways.

Results from brainstem stimulation experiments suggest that catecholaminergic axons in the dorsal horn may operate via two separate mechanisms. Stimulation in the region of the nucleus locus coeruleus (Hodge et al., 1983; Liu and Zhao, 1992; Mokha et al., 1985; Zhao and Duggan, 1988), the A1-C1 group (Liu and Zhao, 1992) and the dopaminergic A11 cell group (Fleetwood-Walker et al., 1988) has been shown to depress nociceptive responses with far greater potency than responses to innocuous

stimuli, while activation in the region of the subcoeruleus-parabrachial nuclei (Girardot et al., 1987) and Kölliker-Fuse nucleus (Zhao and Duggan, 1988) produces a non-selective inhibition of sensory transmission. In the cat, the Kölliker-Fuse nucleus is the primary source of CA in the lumbar spinal cord (Stevens et al., 1982). Hence, most of the terminals examined in the present study will have originated from this nucleus. In this context, our present findings provide a good morphological correlate for the non-selective inhibition of nociceptive and non-nociceptive responses that Kölliker-Fuse nucleus stimulation evokes in the cat (Zhao and Duggan, 1988).

(B). SPINOCERVICAL TRACT (SCT) NEURONS.

These experiments indicate that cells of origin of the SCT, when compared with PSDC neurons, are not a major projection target of descending CA-containing axons. Over 60% of PSDC neurons were found to receive contacts from DBH-positive axons on their somata and proximal dendrites, with some cells receiving up to 22 contacts. In contrast, only 13% of SCT cells were innervated by DBH-immunoreactive axons and the maximum number of contacts observed was 7. Owing to a shortage of time, no ultrastructural studies were made of contacts between CA-containing axons and SCT cells. Hence, it is not known what number, if any, were regions of synaptic input.

Ionophoretic studies in cats have shown that dopamine

and noradrenaline (Fleetwood-Walker et al., 1985, 1988) selectively inhibit the responses of SCT cells to noxious stimuli, and the authors suggested that the most likely explanation for this effect was that the CA's were acting at a site remote from the SCT cell. The present results appear to support this hypothesis, since only a small minority of SCT cells received contacts from CA-containing nerve terminals.

(C). LATERAL CERVICAL NUCLEUS (LCN) NEURONS.

The results from this part of the study show that the feline LCN is innervated by CA-containing axons and varicosities and that these structures are present throughout the entire nucleus. The density of this innervation is similar to that found in the superficial dorsal horn with the same antiserum (see above).

Ultrastructural analysis of TH-IR profiles throughout the LCN confirmed that these structures were axon terminals and most of these (87%) formed synaptic junctions. The postsynaptic targets of CA-containing boutons were dendrites and somata, but not other axon terminals, which suggests that CA neurotransmitters act upon local circuit neurons and/or projection neurons within the LCN, but do not directly influence the axons of SCT cells which terminate there. The absence of axo-axonic synaptic junctions is in keeping with the results of previous fine structural studies of the LCN (Broman and Westman, 1988; Maxwell et al., 1989; Svensson et al.,

1987; Westman, 1968b).

It has been shown that the internuncial cells of the LCN are small (mean diameter 15μm) and located in the medial third of the nucleus (Flink and Westman, 1986), whilst the thalamic and mesencephalic projection neurons of the LCN are large (mean diameter 30μm) and are most numerous in the lateral two-thirds of the nucleus (Berkley et al., 1980; Craig and Burton, 1979; Flink and Westman, 1986). Although the postsynaptic target neurons of TH-IR axons were not identified in the present study, labelled terminals were found in synaptic contact with neuronal somata exhibiting typical features of local circuit cells (Fig.26) and ascending projection neurons (Fig.27E,F). The majority of axo-dendritic synapses was found on medium-sized dendrites, but contacts upon large and small dendrites were also found. It appears, therefore, that contacts are found throughout the dendritic arbors of LCN neurons.

The origin of CA-containing axons in the LCN is unknown, but it is likely to be from neurons with cell bodies located in the brain (Clark and Proudfit, 1991a,b, 1993; Ross et al., 1981; Stevens et al., 1982; Westlund et al., 1983,1984). Retrograde labelling studies (Craig, 1978) have suggested that some of the CA-containing axons in the LCN may be derived from the lateral reticular nucleus and the nucleus tractus solitarius (locations of the A1 & A2 noradrenergic and C1 and C2 adrenergic nuclei of the medulla). However, the numbers of neurons labelled from the LCN were quite small and it seems

unlikely that the catecholaminergic innervation to the LCN is derived entirely from these loci. Most CA-containing axons in the spinal dorsal and ventral horns are derived from cells located in the A5-A7 groups of the pons (Clark and Proudfit, 1991a,b, 1993; Stevens et al., 1982; Westlund et al., 1983, 1984), but it is not known if these nuclei also project to the LCN.

The LCN is a synaptic stage in the spino-cervico-thalamic pathway (Craig et al., 1987; Ha and Liu, 1966; Svensson et al., 1985a) and, accordingly, its major afferent input is from the SCT (Brown et al., 1980a; Craig, 1978; Enevoldson and Gordon, 1989b). The response properties of LCN neurons (Brown et al., 1989) are very similar to those of SCT cells (Brown, 1971; Brown and Franz, 1969). Little is known about the effects of descending pathways, including those containing CA's, upon sensory transmission through the LCN. Dostrovsky (1984) has shown that electrical stimulation of the nucleus raphe magnus, periaqueductal grey and several other brainstem nuclei can inhibit the cutaneous responses of LCN neurons but, since stimulation from these regions only weakly affected excitation produced by stimulating the dorsolateral funiculus, it was concluded that these descending pathways act upon inputs to SCT cells. Craig (1978) has demonstrated the existence of descending connections to the LCN from several medullary nuclei, including the regions of the A1-C1 and A2-C2 CA nuclei, but nothing is known about the effects these

descending noradrenergic or adrenergic pathways have upon LCN neurons. CA-containing axons in the spinal dorsal horn have an inhibitory action upon sensory transmission (Engberg and Ryall, 1966; Fleetwood-Walker et al., 1985, 1988; Headley et al., 1978), and it might be expected that CA-containing axons in the LCN would have the same effect.

(3). ANALYSIS OF NEUROPEPTIDE Y-IMMUNOREACTIVE PROFILES IN THE DORSAL HORN.

It has been suggested, on the basis of the extensive co-localization of NPY with catecholamines in spinally projecting brainstem nuclei (Everitt et al., 1984), that many fibres in the dorsal horn may contain both neurotransmitters (Duggan et al., 1991). Therefore, an examination was made of axons containing neuropeptide Y, to assess whether co-storage between catecholamines and this peptide is likely in the spinal dorsal horn.

(a). **Light microscopical observations.**

The results show that NPY-containing axons and terminals in the cat lumbosacral spinal dorsal horn are concentrated within the superficial layers (laminae I and II). This corresponds to observations made by other authors in rat (Chronwall et al., 1985; De Quidt and Emson, 1986; Gibson et al., 1984; Hökfelt et al., 1981; Hunt et al., 1981b; Sasek and Elde, 1985; Wakisaka et

al., 1991) and a number of other species (Allen et al., 1984; Blessing et al., 1987; Gibson et al., 1984; Merighi et al., 1990), but is not entirely consistent with what has been previously described for cats (Gibson et al., 1984; Krukoff, 1987). In these latter studies, the authors concluded that there was a heavy NPY innervation to lamina III in the cat, but examination of their figures shows that only a few NPY terminals are located in that region. The heaviest innervation, in accordance with the present findings, was in the superficial layers.

In transverse sections of laminae I and II, NPY-containing terminals were present predominantly as single, isolated punctate structures and axonal strands were only occasionally observed. This description of NPY-IR terminals is in accord with previous studies (Allen et al., 1984; Hökfelt et al., 1981; Hunt et al., 1981b; Sasek and Elde, 1985). In sagittal sections, however, superficially located NPY axons were often long and varicose. Sugiura et al. (1986) have shown using an intracellular staining technique that cutaneous unmyelinated (C) fibres, within laminae I and II, often distribute as long, rostrocaudally oriented strands. This similar orientation of NPY-containing axons and fine primary afferent axons could imply a close association between the two groups. The dendritic arbors of many neurons in this region are also oriented rostrocaudally (Gobel, 1978; Gobel et al., 1980), which provides an opportunity for NPY-IR fibres to form synaptic contacts

with them.

(b). **Ultrastructural observations.**

Serial section analysis of NPY-containing profiles throughout laminae I-VI of the dorsal horn confirmed that the majority of structures were axon terminals, although a few structures were observed that were equivocal. Axon terminals immunostained for NPY contained two distinct types of vesicle. Most vesicles were small, clear and usually round, but occasional large dense-core vesicles were found that were immunoreactive. This corresponds to previous descriptions of NPY-positive terminals in the cortex (Aoki and Pickel, 1990; Hendry et al., 1984), striatum (Aoki and Pickel, 1990), hypothalamus (Pelletier et al., 1984) and the thoracic intermediolateral cell column (Llewellyn-Smith et al., 1990).

Almost all NPY-IR axon terminals (95%) formed synaptic specializations. Most synaptic junctions were formed on the dendrites and somata of dorsal horn neurons (73% of synapses throughout laminae I-VI). This observation is compatible with a postsynaptic action on local circuit neurons and/or projection neurons. The majority of axo-dendritic synapses was found on medium-sized dendrites but contacts on large calibre dendrites and small diameter dendrites were not uncommon. It appears, therefore, that contacts are found throughout the dendritic arbors of dorsal horn cells.

Many NPY-IR terminals within all six dorsal horn laminae formed synaptic junctions with other axon

terminals, and the ultrastructural appearance of these synaptic junctions was similar to that of axo-axonic synapses previously described in the spinal cord (McLaughlin et al., 1975). In laminae I-III, NPY-containing nerve terminals were sometimes presynaptic to the large central boutons of synaptic glomeruli. Almost all central glomerular boutons disappear following dorsal rhizotomy (Knyihar-Csillik et al., 1982), therby demonstrating that they are primary afferent in origin. Hence, the present study suggests that NPY-containing axons in the dorsal horn regulate spinal sensory transmission through a presynaptic action upon primary afferent terminals.

Although most of the NPY-IR profiles examined in this study (194/208) were typical axon terminals, 14 vesicle-containing structures were examined that were equivocal. These profiles had some features of vesicle-containing dendrites (Ralston, 1971), but no obvious NPY-IR cell bodies or dendrites were observed in this study with the light microscope. Since it was not possible to trace any of these vesicle-containing profiles back to the point where organelles such as rough endoplasmic reticulum were visible, no definitive statements can be made about their nature.

(c). **Origins of NPY-containing axons in the dorsal horn.**

Immunocytochemical studies in the rat, guinea-pig, horse, pig and marmoset (Gibson et al., 1984; Hökfelt et

al., 1981; Merighi et al., 1990; Wakisaka et al., 1991) have produced no evidence for the presence of NPY in dorsal root ganglion cells, despite the fact that other peptides such as substance P, calcitonin gene-related peptide and galanin were readily detected (Hökfelt et al., 1981; Merighi et al., 1990), and it is now generally accepted that NPY is not present in these structures, at least in the species noted above (Weihe, 1990). Furthermore, no changes are seen in the density of NPY-immunostaining in the dorsal horn following procedures which destroy the primary afferent input to the dorsal horn of rats (Gibson et al., 1984; Hökfelt et al., 1981). Therefore, NPY axons in the dorsal horn of most species are derived from spinal and/or supraspinal neurons. In the cat, however, a small proportion of dorsal horn NPY-containing fibres may be derived from primary afferent neurons. Lindh et al. (1989) have shown that a few dorsal root ganglion cells in the cat contain NPY and NPY-positive fibres have been observed in Lissauer's tract and in the dorsal roots in this species (Gibson et al., 1984).

An obvious potential source of NPY terminals in the dorsal horn are neurons with cell bodies located in the brain. Immunocytochemical studies have shown that NPY-IR perikarya found in several brain regions which are known to project to the spinal cord, including the cortex and the brainstem noradrenergic nuclei (Adrian et al., 1983; Allen et al., 1983; Blessing et al., 1986,1987; Chronwall et al., 1985; Coveñas et al., 1990; DeQuidt and Emson,

1986; Everitt et al., 1984; Halliday et al., 1988; Hendry et al., 1984; Hökfelt et al., 1983a,b; Holets et al., 1988; Hunt et al., 1981a; Lundberg et al., 1980; Sawchenko et al., 1985; Yamazoe et al., 1985). However, cortical NPY perikarya correspond to the non-pyramidal neurons which are intrinsic or local circuit neurons (Hendry et al., 1984) and, of the noradrenergic cell groups, only a few NPY-neurons located in the locus coeruleus (A6) project to the dorsal horn (Holets et al., 1988). It has been suggested, on the basis of the extensive co-localization of NPY with catecholamines (CA'S) in spinally projecting brainstem nuclei, that many fibres in the dorsal horn may contain both neurotransmitters (Duggan et al., 1991). However, this does not seem to be the case. For instance, the caudal distribution and density of NPY-IR fibres in the dorsal horn is not visibly altered following complete section or hemisection of the spinal cord (Hökfelt et al., 1981; Hunt et al., 1981b), whereas CA levels fall to zero (Carlsson et al., 1964; Dahlström and Fuxe, 1965; Magnusson and Rosengren, 1963). Furthermore, double-labelling studies have found no evidence for NPY terminals in the dorsal horn that co-contain CA's (Blessing et al., 1987). The present results also suggest that there is, at best, only limited co-localization of CA's and NPY in the dorsal horn. For instance, there are notable differences in the morphology and distribution of axonal swellings stained for the two

neurotransmitters. Many NPY boutons are highly flattened, whereas CA-containing terminals are almost always round or oval. Furthermore, the catecholaminergic innervation to the superficial laminae is not as great as that observed with NPY, nor is it as punctate. In addition, CA-containing axons within the deeper dorsal horn are not organized into the prominant fibre bundles observed with NPY. Further, ultrastructural studies have shown that NPY-IR boutons form axo-axonic associations, whereas CA-containing nerve terminals do not. Thus, the axonal networks seem to have different origins within the central nervous sytem; dorsal horn NPY terminals being derived principally from local circuit neurons and CA-containing axons originating from supraspinal sites. However, NPY and CA are extensively co-localized in axon terminals within the thoracic intermediolateral cell column (Blessing et al., 1987; Tseng et al., 1993). These axons are derived mainly from the rostral ventrolateral medulla (C1 CA nucleus) (Blessing et al., 1987; Tseng et al., 1993), and degenerate following cervical cord transection (Hökfelt et al., 1981).

Large numbers of NPY-synthesizing perikarya have been revealed within the spinal cord of rats and cats following the injection of colchicine into the dorsal horn (Hunt et al., 1981a,b) or intrathecally (DeQuidt and Emson, 1986; Krukoff, 1987; Sasek and Elde, 1985). In rats (DeQuidt and Emson, 1986; Hunt et al., 1981a,b; Sasek and Elde, 1985), NPY perikarya were detected in large numbers in laminae I-III throughout the cord. This

finding has recently been confirmed by a sensitive immunostaining procedure which does not require the use of colchicine (Rowan et al., 1993). In cats (Krukoff, 1987), the distribution of NPY perikarya was markedly different; cells were only present caudal to segment T2, where they were concentrated in lamina VII, with additional cells in laminae VI, VIII and IX. In the rabbit (Blessing et al., 1987) and pig (Merighi et al., 1990) a few NPY-positive cell bodies have been observed in laminae I-III of the dorsal horn without the use of colchicine. Rowan et al. (1993) have shown that all NPY-IR neurons in the rat spinal cord co-contain GABA. In view of the fact that NPY in the dorsal horn originates mainly from spinal neurons and, assuming that this co-storage pattern also exists in the cat, it is possible that the majority of NPY-IR nerve terminals examined in this study were also GABAergic. Accordingly, NPY-positive boutons, like those immunostained for GABA (Barber et al., 1978; Basbaum et al., 1986; Magoul et al., 1987; Maxwell et al., 1990a,b; Maxwell and Noble, 1987; McLaughlin et al., 1975; Merighi et al., 1989; Todd and Lochhead, 1990), were presynaptic to other axon terminals, including primary afferent terminals, as well as to dorsal horn neurons.

(d). **Functional considerations.**

The presence of high numbers of NPY axons and NPY receptors (Kar and Quirion, 1992) in the superficial

dorsal horn suggests that NPY may regulate sensory information flowing into the central nervous system. Furthermore, since this region receives much of the cutaneous afferent input from Aδ and C nociceptors (Light and Perl, 1979b; Sugiura et al., 1986), and little input from low threshold afferents (Brown, 1981; Light and Perl, 1979b; Sugiura et al., 1986), this regulation may be most prominant upon activity generated by noxious stimuli. It is significant, therefore, that NPY has been shown to produce analgesia in conscious rats following intrathecal administration (Hua et al., 1991).

There are several lines of evidence which suggest that the analgesic effect of NPY may be brought about, at least in part, by a presynaptic inhibitory action upon the terminals of nociceptive primary afferent axons within the dorsal horn. For instance, it has been demonstrated that rat sensory neurons in culture possess unusually high concentrations of specific binding sites for NPY and that activation of such receptors inhibits the K^+ stimulated release of substance P from these cells (Bleakman et al., 1991; Walker et al., 1988). A similar effect has been demonstrated in the cat dorsal horn *in vivo* (Duggan et al., 1991). Furthermore, the number of NPY binding sites in the superficial dorsal horn is dramatically reduced when the primary afferent input to this region is removed by dorsal rhizotomy or neonatal capsaicin treatment (Kar and Quirion, 1992). The present ultrastructural observations are also consistent with a presynaptic inhibitory action of NPY, since boutons

immunoreactive for NPY often synapse onto other axons, including the central boutons of glomeruli. Moreover, the presence of NPY in axons which are presynaptic to central boutons, some of which contain substance P (Riberio da Silva et al., 1989), may help explain the observation that when NPY is injected into lamina II it reduces the release of substance P from C fibres (Duggan et al., 1991).

At present little is known about the effects of NPY upon dorsal horn neurons, and it is not known if the axo-dendritic and axo-somatic synaptic junctions observed in the present study are excitatory or inhibitory. However, since GABA is likely to be co-localized with NPY in these terminals, they would be predicted to be inhibitory.

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Appendix.

Some of the results presented in this thesis have been published as follows:

Doyle, C.A. & Maxwell, D.J. (1990). Synaptic organization of tyrosine hydroxylase-immunoreactive boutons in the lumbosacral spinal cord of cat. J. Physiol. 426, 55P.

Doyle, C.A. & Maxwell, D.J. (1991). Catecholaminergic innervation of the spinal dorsal horn: a correlated light and electron microscopic analysis of tyrosine hydroxylase-immunoreactive fibres in the cat. Neuroscience 45, 161-176.

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Reprints of these publications are presented overleaf.

[*From the Proceedings of the Physiological Society*, 30–31 *March* 1990
Journal of Physiology, **426**, 55*P*, 1990]

Synaptic organization of tyrosine hydroxylase-immunoreactive boutons in the lumbosacral spinal cord of the cat

By C. A. Doyle and D. J. Maxwell. *Department of Preclinical Veterinary Sciences, University of Edinburgh, Edinburgh EH9 1QH*

Catecholamine-containing axons in the lumbosacral dorsal horn of the spinal cord originate from supraspinal neurones located in nuclei of the brainstem (Westlund *et al.* 1984). Activation of these descending systems or ionophoretic application of catecholamines into the dorsal horn modifies response characteristics of some dorsal horn neurones and, in particular, may depress activity generated by noxious stimuli (Besson & Chaouch, 1987). However, the mechanisms underlying these inhibitory processes remain obscure.

We have investigated the synaptic organization of presumed catecholamine-containing boutons in the dorsal horn in a correlated light and electron microscopic study of axons labelled with an antiserum specific for tyrosine hydroxylase (van den Pol *et al.* 1984). Three adult cats were anaesthetized with sodium pentobarbitone (40 mg/kg, I.P.) and fixed by trans-cardial perfusion. Sections of lumbosacral (S1–L7) spinal cord were processed immunocytochemically and prepared for combined light and electron microscopic analysis.

Numerous varicose-immunoreactive axons were observed with the light microscope in laminae I–IV; correlated ultrastructural analysis confirmed that these varicosities corresponded to synaptic boutons (mean diameter $\pm$ S.D. = $1{\cdot}04 \pm 0{\cdot}3$ μm). In all, 26 synaptic boutons were examined through serial sections. Boutons contained agranular and dense-core vesicles and formed small (0·36 μm) symmetrical synaptic junctions with dendrites (range = 0·25–3·04 μm) and somata of dorsal horn neurones.

The evidence reported in this study establishes for the first time at the ultrastructural level that descending catecholaminergic systems form synapses with neurones in laminae I–IV and thus are likely to influence sensory transmission in the dorsal horn through postsynaptic actions on dorsal horn neurones.

Supported by the MRC.

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Neuroscience Vol. 45, No. 1, pp. 161–176, 1991
Printed in Great Britain

0306-4522/91 $3.00 + 0.00
Pergamon Press plc

CATECHOLAMINERGIC INNERVATION OF THE SPINAL DORSAL HORN: A CORRELATED LIGHT AND ELECTRON MICROSCOPIC ANALYSIS OF TYROSINE HYDROXYLASE-IMMUNOREACTIVE FIBRES IN THE CAT

C. A. DOYLE* and D. J. MAXWELL

Department of Preclinical Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, U.K.

Abstract—The ultrastructural organization of presumed catecholamine-containing boutons, in the dorsal horn of the cat lumbosacral spinal cord, was examined in an immunocytochemical study using an antiserum against tyrosine hydroxylase. The study was restricted to the first four laminae of Rexed. Light microscopic inspection revealed numerous, varicose, tyrosine hydroxylase-immunoreactive axons throughout this region of the spinal cord. Within laminae I and II the fibres exhibited a prominent rostrocaudal orientation, while in laminae III and IV they were organized predominantly dorsoventrally. Correlated ultrastructural analysis confirmed that these varicosities were synaptic boutons. Forty-five of these structures were examined through serial sections and they were found to form symmetrical (Gray type II) synaptic junctions with dendrites (95%) and somata (5%). Immunoreactive boutons were not observed to be either presynaptic or postsynaptic to axon terminals.

These findings suggest that catecholamines within the spinal dorsal horn act through a postsynaptic action upon dorsal horn neurons.

Catecholamine-containing nerve terminals have been shown to innervate the spinal gray matter of a number of species, including the rat,[11,32,82,90] cat,[19,56] opossum[60,61,70] and primates.[16,91] Although these structures are distributed throughout the gray matter, they are particularly abundant within the superficial layers (laminae I and II) of the dorsal horn.

Lesion studies in combination with biochemical or histochemical analysis, together with retrograde transport techniques have demonstrated that these terminals arise from neurons whose cell bodies are located in supraspinal sites (see Ref. 92 for review). The pontine locus coeruleus (A6), nuclei of the A7 group, A5 and A4 groups all send descending noradrenergic projections to the dorsal horn. The dopaminergic innervation to the spinal cord is thought to be derived solely from the diencephalic A11 cell group.[8,45,83,84] Evidence suggests that in mammals the axons of descending catecholaminergic neurons course through the dorsolateral funiculus and ventrolateral and ventral quadrants before terminating in the dorsal horn.[3,11,19] Recently, however, it has been proposed that most coerulospinal fibres in the rat may descend through the gray matter.[32]

Behavioural studies have shown that intrathecal administration of sympathomimetic drugs produces potent analgesia.[48,50–54,72] Furthermore, ionophoretic application of catecholamines depresses the activity generated in dorsal horn neurons following peripheral stimulation.[4,20,25,29,30,41,46,80] This is consistent with results from brainstem stimulation studies, in which electrical activation of A6[42,65,77,78,97] and A7 [35,97] noradrenergic cell groups and the dopaminergic A11 cell group[29] produces similar effects.

A number of authors have proposed that the predominant action of catecholamines in the spinal cord is to selectively inhibit responses to noxious stimuli.[4,20,29,30,41] However, others have found that catecholamines depress activity generated by both high and low threshold stimuli.[46,80] Electrical activation of the locus coeruleus[42,65,97] and the dopaminergic A11 cell group[29] has been found to depress nociceptive transmission much more powerfully than responses to innocuous cutaneous stimulation, inferring some degree of selectivity in the actions of these catecholaminergic nuclei. In contrast, stimulation of the A7 cell group produced a non-selective regulation of sensory transmission.[46,97]

The circuitry underlying these effects is very poorly understood, but both presynaptic[13,17,40,49,55,94] and postsynaptic[4,25,48,68] mechanisms have been suggested. The present study was prompted by the need to improve our understanding of the mechanisms through which catecholaminergic neurons terminating within the spinal dorsal horn regulate somatosensory transmission in the cat.

Tyrosine hydroxylase (TH) is the enzyme which converts tyrosine to dihydroxyphenylalanine

*To whom correspondence should be addressed.

Abbreviations: DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase; 5-HT, 5-hydroxytryptamine, serotonin; NA, noradrenaline; NDS, normal donkey serum; PBS, phosphate-buffered saline; TH, tyrosine hydroxylase.

(DOPA), and is the first enzyme in the catecholamine biosynthetic pathway. Therefore, it is an endogenous enzyme of dopaminergic, noradrenergic and adrenergic neurons. To characterize the ultrastructural organization of the catecholaminergic innervation to the spinal cord, we have examined catecholaminergic nerve fibres in a correlated light and electron microscopic analysis, using TH as an immunocytochemical marker. The ultrastructural analysis was restricted to the first four laminae of the dorsal horn. A preliminary abstract of some of these findings has been published.[24]

EXPERIMENTAL PROCEDURES

Fixation of tissue

Five cats were anaesthetized with sodium pentobarbitone (40 mg/kg, i.p.), and perfused through the heart with warm saline (37°C) containing 100 U/ml heparin and 0.1% sodium nitrite. This solution was delivered at a pressure of 120 mmHg and, once the blood had cleared, 1000 ml of warm fixative (37°C) was introduced at the same pressure. The fixative consisted of 4% paraformaldehyde, 0.1% glutaraldehyde and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.4. A further 2000 ml of fixative was delivered at 4°C.

Following perfusion, the lumbosacral region (L7–S1) of the spinal cord was removed and kept in the same fixative (with the omission of glutaraldehyde) for a further 6 h. The blocks were washed and stored overnight in phosphate-buffered saline (PBS). Tissue from the striatum was also removed to assess the immunocytochemical procedure (see later). Transverse and sagittal sections (40 μm) of spinal cord were cut with a Vibratome, and rinsed overnight in several changes of PBS to ensure complete removal of fixative prior to incubation with the primary antiserum. Sections were also cut from the striatum.

Immunocytochemical procedure

Vibratome sections were immersed in 10% normal donkey serum (NDS) with 0.01% sodium azide in PBS for 30 min to reduce nonspecific (background) staining by antisera. For immunostaining, sections were incubated in the TH antiserum diluted 1:1000 for 18–24 h at 4°C. The TH antiserum employed in this study was raised by van den Pol *et al.*, and its characteristics and specificity have been described elsewhere.[89] TH was visualized in test sections using the avidin–biotin–peroxidase complex (ABC) technique.[47] Following treatment with the primary antiserum the sections were incubated for 45 min in anti-rabbit biotinylated antibody (Amersham) diluted 1:100 at room temperature, followed by 15 min in streptavidin peroxidase complex (Amersham) diluted 1:300 at room temperature. The tissue was washed between each step in PBS and dilutions were made in PBS containing 1% NDS. The presence of peroxidase was visualized by reacting with 3,3′-diaminobenzidine (DAB) and hydrogen peroxide.

Electron microscopy

Following the DAB reaction, sections were postfixed in 1% osmium tetroxide for 1 h and dehydrated through a series of ethanol solutions. "*En bloc*" staining was performed with a 1% solution of uranyl acetate in 70% ethanol. After dehydration they were cleared in propylene oxide, flat-embedded in Durcupan between plastic foils and polymerized at 60°C for 48 h. When polymerization was complete, the sections were examined then photographed or drawn with the aid of a drawing tube.

Sections were subsequently attached to Durcupan blocks and thin sections cut on an ultramicrotome. Ribbons of serial thin sections were collected on single-slot copper grids coated with Formvar and stained with Reynold's lead citrate for 2–3 min. Immunostained axons within the dorsal horn of the spinal cord were examined with the electron microscope through the series in order to determine the synaptic arrangements that they formed. Bouton diameters were measured using a Reichert videoplan system from micrographs where the profiles were at their largest.

Effects of fixation and Triton X-100

Two techniques were employed to improve accessibility of the antisera to catecholaminergic fibres. (1) Berod *et al.*[6] have shown that increasing the pH of fixative enhanced TH immunohistofluorescence in the caudate nucleus. Therefore one of the cats was perfused with a solution of 4% paraformaldehyde in 0.1 M borate buffer at pH 9.0. (2) In a second series of experiments, penetration of immunoglobulins was increased by incubation of the sections in up to 0.3% Triton X-100 for 1 h, prior to incubation with the TH antiserum.

Controls

TH antiserum was omitted from the incubation medium in control experiments. In addition, sections from the striatum were incubated in primary antiserum and processed identically to the spinal cord test sections. This region possesses a dense catecholaminergic innervation,[58] and is known to react strongly with this antiserum.[31] These sections were used for comparative purposes.

RESULTS

Light microscopic observations of tyrosine hydroxylase-immunoreactive axons

Nerve fibres exhibiting TH immunoreactivity were found in all laminae of the spinal dorsal horn, but within each lamina differences were noted with respect to the density and trajectory of the axons and varicosities.

In transverse sections, TH-immunoreactive fibres were most abundant in the medial part of laminae I, II and IV (Fig. 1). Additional axons were seen to form a dense plexus around the central canal. Innervation in the remainder of the gray matter, including the ventral horn, was relatively sparse. Within laminae I and II of the dorsal horn, TH immunoreactivity

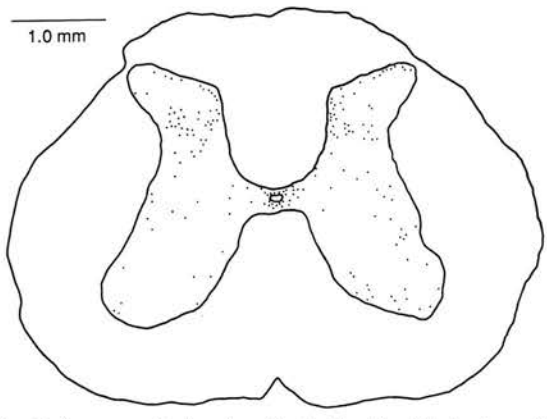

Fig. 1. A composite drawing illustrating the distribution of the TH-immunoreactive axons throughout five 40-μm transverse sections of cat lumbosacral (L7–S1) spinal cord. Note the particularly high density of fibres in laminae I, II and IV and around the central canal in lamina X.

was observed primarily as isolated punctate profiles or short axons with few "*en passant*" varicosities (Fig. 3B). Long strands of catecholaminergic axons were occasionally seen travelling in the transverse plane (Fig. 3B, C). In lamina IV, TH immunoreactivity was observed primarily as short varicose axons running in both the dorsoventral and mediolateral planes. Throughout laminae I–IV, terminals and varicosities were seen in contact with somata (Fig. 4).

Analysis of the distribution of immunolabelled axons in the sagittal plane revealed large numbers of rostrocaudally orientated axons within laminae I and II (Fig. 2). These axons were most numerous in lamina I. Large numbers of immunoreactive fibres were also found in laminae III and IV, but these structures were predominantly orientated dorsoventrally. In contrast to the large numbers of labelled fibres in the first four laminae of the dorsal horn, the remainder of the gray matter, including the ventral horn, contained very few TH-immunoreactive axons (Fig. 2).

Effects of fixation and Triton X-100

The high pH fixation regime recommended by Berod *et al.*[6] only marginally improved the intensity of immunolabelling within the spinal dorsal horn. In addition, this fixation produced poor ultrastructural preservation.

Pre-treatment of tissue sections with Triton X-100 greatly enhanced the density of immunolabelling. Particularly noticeable were the large numbers of rostrocaudally orientated fibres revealed within laminae I, II and the outer portion of lamina III. These fibres were highly varicose and could often be followed for several millimetres (Fig. 5). Most immunolabelled axons ran parallel with the dorsal border of the dorsal horn. The rostrocaudal orientation was most prominent in lamina I. In lamina II and the outer portion of lamina III the predominant orientation of the TH-immunoreactive axons was still rostrocaudal, but many axons travelled at an angle to the dorsal border of the dorsal horn, and some axons followed a highly curved path (Fig. 5B). Frequently, these axons ran for long distances without branching (Fig. 5A). In the inner portion of laminae III and IV the fibres travelled dorsoventrally. Treatment with Triton X-100 produced a marked deterioration in tissue preservation, and these sections were not used for ultrastructural analysis.

The TH-immunoreactive fibres may be classified into at least two distinctive populations on the basis of the size of their varicosities. The most common type of axon had both large and small varicosities intermingled along its length (Fig. 6A), while the second, less common type was characterized by small varicosities, which were only slightly larger than their intervaricose axons (Fig. 6B). Pre-treatment with Triton X-100 revealed large numbers of immunoreactive varicosities which were in contact with neuronal somata (Fig. 5).

Since treatment with Triton X-100 results in tissue deterioration, these sections were not used for ultrastructural analysis.

Controls

Sections which had been incubated in a medium from which the primary antiserum had been omitted displayed no immunolabelling. Sections from the striatum which had been treated with the TH antiserum displayed an intense immunoreaction.

Ultrastructural observations of tyrosine hydroxylase-immunoreactive axons

In all, 45 boutons exhibiting TH immunoreactivity were examined through serial sections with the electron microscope. Terminals were predominantly round or oval in shape and varied in size from 0.40 to 1.83 μm in diameter. A frequency distribution histogram of bouton diameters is shown in Fig. 10A. The majority (85%) of profiles fell within the range 0.6–1.2 μm (0.94 μm mean diameter). Catecholaminergic boutons characteristically formed single synapses on dendrites (95% of synapses) or neuronal

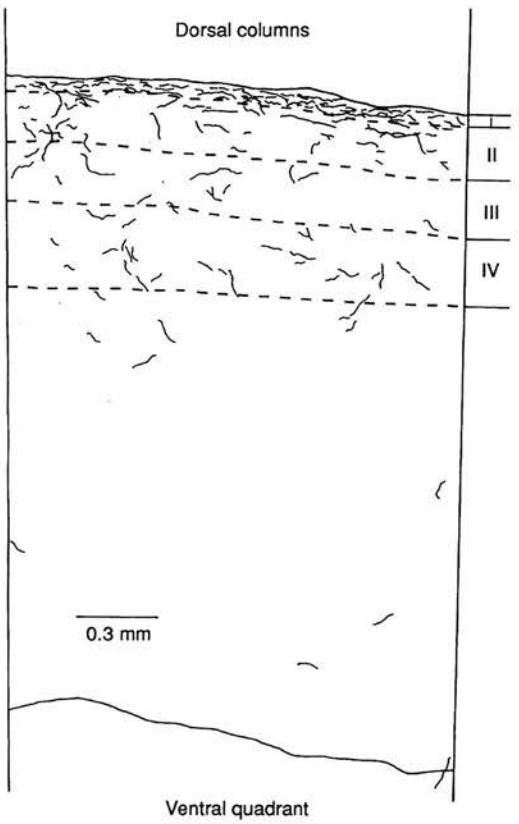

Fig. 2. A composite drawing illustrating the distribution of TH-immunoreactive axons throughout four 40-μm sagittal sections of cat lumbosacral (L7–S1) spinal cord. Note the very dense innervation in laminae I–IV compared to the rest of the gray matter. In lamina I and the outer region of lamina II the fibres exhibit a prominent rostrocaudal orientation. In laminae III and IV this orientation changes, and most axons are organized dorsoventrally.

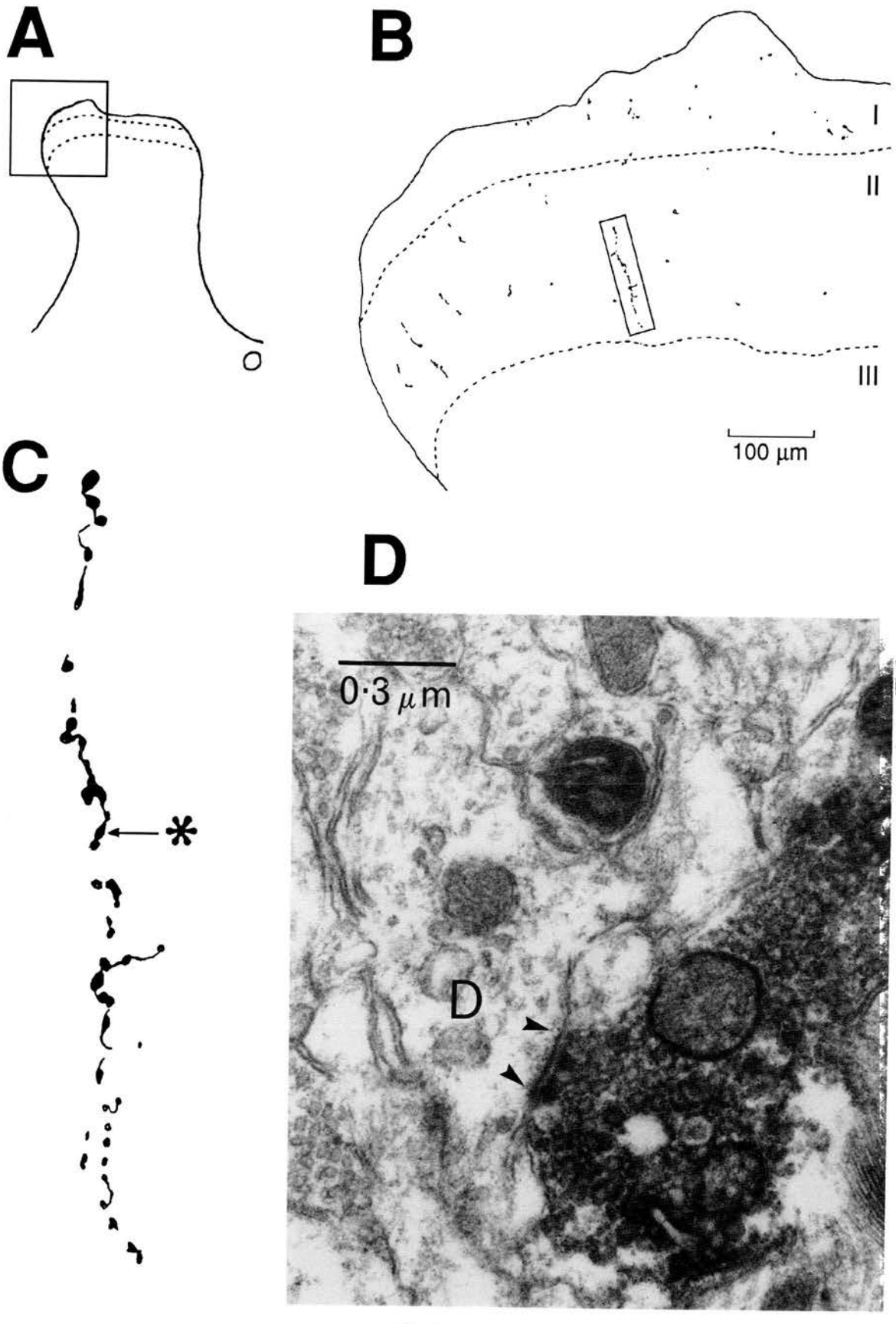

Fig. 3.

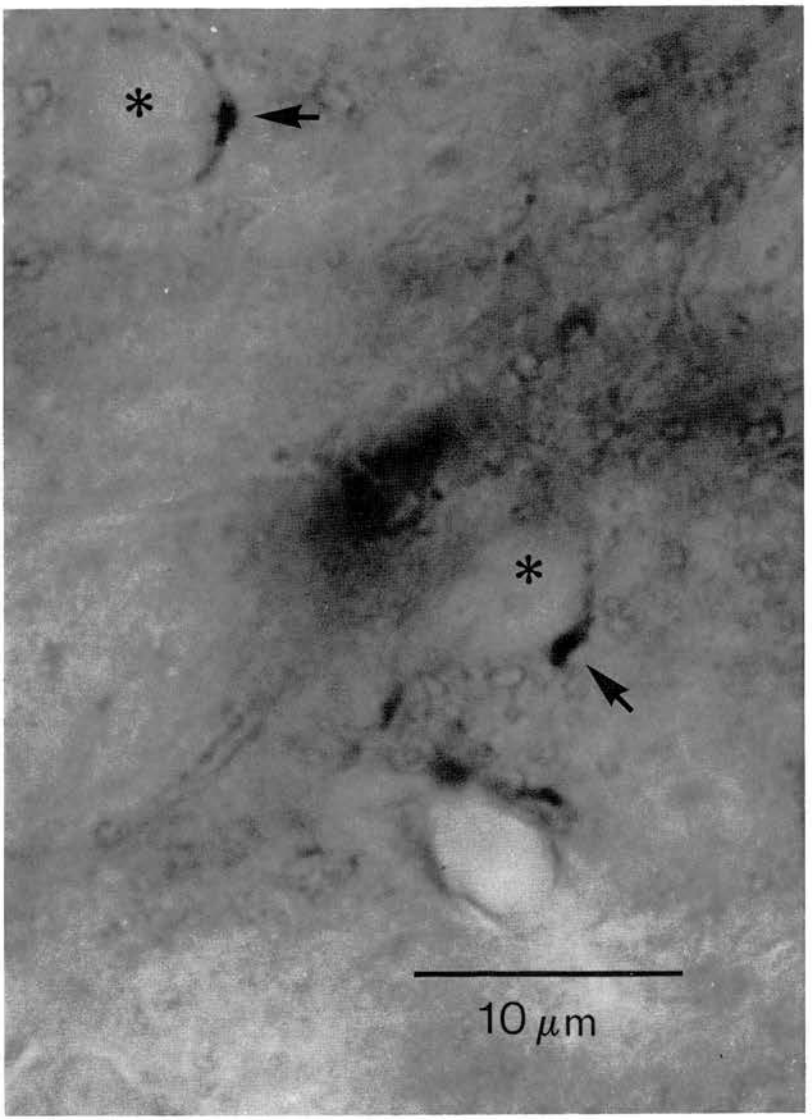

Fig. 4. A light micrograph showing two TH-immunoreactive terminals (arrows) in lamina II contacting small neuronal cell bodies (asterisk).

cell bodies (5% of synapses). They were not observed to form axo-axonic synapses, although they were occasionally found closely apposed to other unlabelled vesicle-containing structures. Intact boutons when followed through serial sections invariably formed synaptic specializations. However, it was often difficult to demonstrate the presence of an active zone if the bouton was disrupted. The boutons were characteristically packed with small, irregularly shaped, agranular vesicles (Figs 3D, 7–9) together with several dense-core vesicles (Fig. 9). The possibility that some dense-core vesicles were immunoreactive for TH was difficult to assess because of the intensity of the electron-dense reaction product. The boutons usually also contained at least one mitochondrion. Synaptic junctions formed by these axonal

Fig. 3. (A) An outline of the dorsal horn of the L7–S1 region of the spinal cord in transverse section. Higher magnification of the area enclosed by the box is shown in B. The distribution of TH-immunoreactive axons within laminae I and II of this region is illustrated for a single 40-μm section. In this particular section the density of immunolabelling is similar for both superficial laminae. Surrounded by a small box is an immunolabelled fibre travelling in the dorsoventral plane. The highly varicose appearance of this axon is shown at higher magnification in C. The varicosity marked by an asterisk is shown in an electron micrograph (D). This bouton forms a symmetrical (Gray type II) synaptic junction (between the arrowheads) with a medium-sized dendrite (D).

terminals were of the symmetrical type (Gray type II), and were small (mean $\pm$ S.D. $= 0.33 + 0.10\,\mu$m), when compared to the diameter of the bouton. The immunoreactive material within labelled boutons was most obvious along vesicle membranes and mitochondrial outer membranes (Figs 8, 9). It is possible, therefore, that the presynaptic densities were exaggerated by the immunoreaction.

Axo-dendritic synapses were found throughout laminae I–IV of the dorsal horn. Immunolabelled boutons formed synapses most commonly with medium-sized (0.5–2.0 μm diameter) dendrites

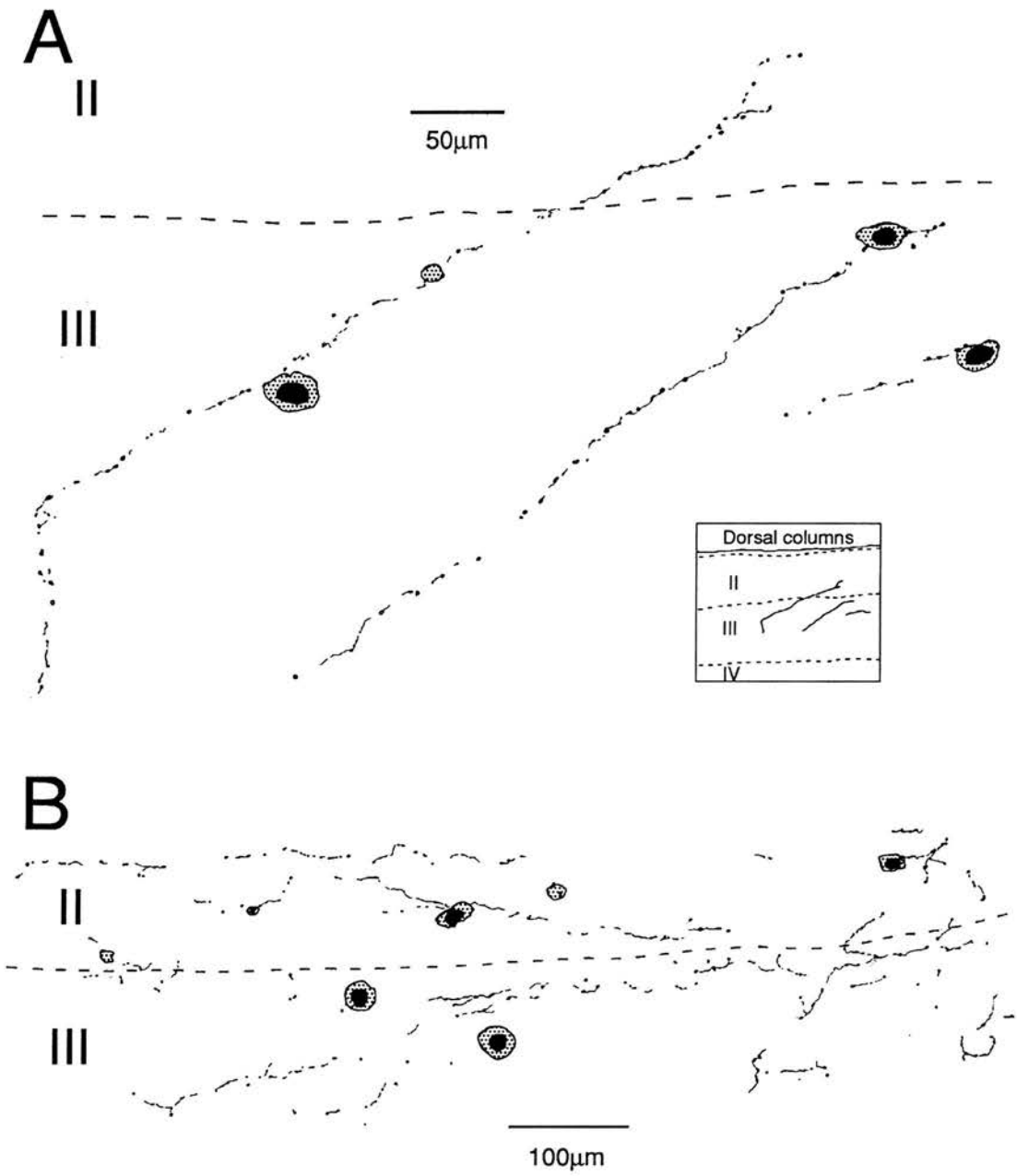

Fig. 5. A pair of drawings, constructed from sagittal sections treated with Triton-X 100, illustrating the morphology and patterns of distribution of TH-immunoreactive axons within lamina II and the outer region of lamina III. (A) TH-immunoreactive axons travel for considerable distances in a rostrocaudal direction without branching. Most fibres are characterized by regularly spaced varicosities. (B) Typical features of immunolabelled axons at the lamina II–III border. Many fibres run rostrocaudally while others travel at an angle to the laminar boundary. Further axons follow a highly curved path. Throughout this region, immunolabelled axons are found in contact with neuronal cell bodies.

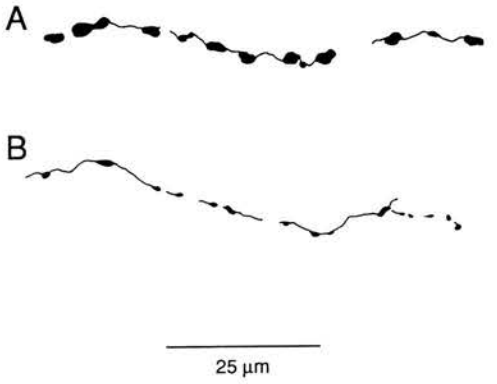

Fig. 6. Drawings of TH-immunoreactive axons typical of those found in the first four dorsal horn laminae. At least two different morphological types of axon can be distinguished by the dimensions of the varicosities which occur along the axonal strands. Axon type B is fairly unusual.

(Figs 3D, 8C, D, 10B). Large calibre dendritic shafts ($>2.0\,\mu$m diameter) also received synapses from TH-immunoreactive axonal endings (Figs 7D, 8E, F, 10B), as did very small ($<0.5\,\mu$m diameter) dendrites or spines (Figs 7E, 8A, B, 10B).

Symmetrical axosomatic synapses were present in laminae II, III and IV. No examples were observed in lamina I, but few immunolabelled boutons were sampled in this region. A small immunoreactive bouton in lamina IV in association with a large neuronal perikaryon is shown in Fig. 9B. At higher magnification (Fig. 9C) the prominent active zone is revealed.

DISCUSSION

Characteristics and specificity of the primary antiserum

The primary antiserum used in this study was raised by van den Pol *et al.*, in rabbits, from TH extracted from bovine adrenal glands. The characteristics of this antiserum have been described elsewhere.[89] Briefly, its specificity for TH was determined by precipitation of enzyme extracts from a crude cell homogenate (Western blot); only a single band of 62,000 mol. wt was recognized by this antiserum. Immunohistochemical specificity was confirmed by the abolition of immunostaining following pre-incubation with TH, but not any other antigens. In our study, sections which had been incubated in a medium lacking TH antiserum displayed no immunolabelling, suggesting that the axons observed in the test sections were immunoreactive for TH. The appearance of the catecholaminergic axons observed in the striatal sections was similar to those observed by Freund *et al.*,[31] who used the same antiserum. These two observations, in conjunction with the specificity findings of van den Pol *et al.*,[89] strongly suggest that the structures labelled in the spinal cord were immunoreactive for TH.

Origins of tyrosine hydroxylase-immunoreactive fibres

The original evidence indicating that the catecholaminergic innervation to the spinal cord arose from cell bodies located in supraspinal sites came from observations that catecholamine histofluorescence almost completely disappeared following transection of the cord.[11,19,59] Dahlström and Fuxe[18] mapped the distribution of catecholamine-containing perikarya in the rat brainstem, and it was proposed that the spinal catecholaminergic terminals originate from one or more of these nuclei.[19] Subsequent studies have shown that these terminals originate from cells in the noradrenergic A4–A7 cell groups (see Ref. 92 for review), and the dopaminergic A11 cell group.[8,45,83,84] A spinal projection may also arise from the medullary A1/C1 and A2/C2 cell groups,[19,27,36] although some authors do not support this idea.[90,91]

A chronic spinal cord transection which severs the descending axons would deplete the spinal cord of catecholamines derived from supraspinal sites. However, catecholamine may still be detected caudal to this type of lesion,[11,19,59] but the concentrations are very low, and make only a modest contribution to the spinal cord catecholamine content. In the cat, it is likely that all of the TH-immunoreactive axons found

Fig. 7. Correlated light and electron microscopic analysis of a TH-immunoreactive axon. (A) Location of the axon in lamina II of the dorsal horn. (B) Light microscopic appearance of the immunocytochemically stained axon. (C) A low power electron micrograph of the same fibre. Note the presence of the two boutons marked 1 and 2. (D, E) At higher magnification the symmetrical (Gray type II) synapses formed by these structures are resolved (between the arrowheads). Bouton 1 synapses with a large calibre dendrite (D), while the dendrite (D) contacted by bouton 2 is small.

Fig. 8. Electron micrographs showing TH-immunoreactive terminals making symmetrical synaptic contacts (between the arrowheads) with small (A, B), medium (C, D) and large (F) dendritic profiles. The bouton in F is shown at lower magnification in E (asterisk). At this magnification the very large calibre of this dendrite (D) is revealed. Scale bars for A–D, F = 0.25 μm; for E = 2.0 μm.

Fig. 9. Electron micrographs showing a TH-immunoreactive bouton forming a symmetrical (Gray type II) synaptic junction with a large neuronal perikaryon in lamina IV. (A) Location of the cell body within the dorsal horn. (B) Low power electron micrograph of the bouton (B) apposed to the large soma. Note the presence of the nucleus (Nuc). (C) At higher magnification the prominent active zone is revealed (between the arrowheads).

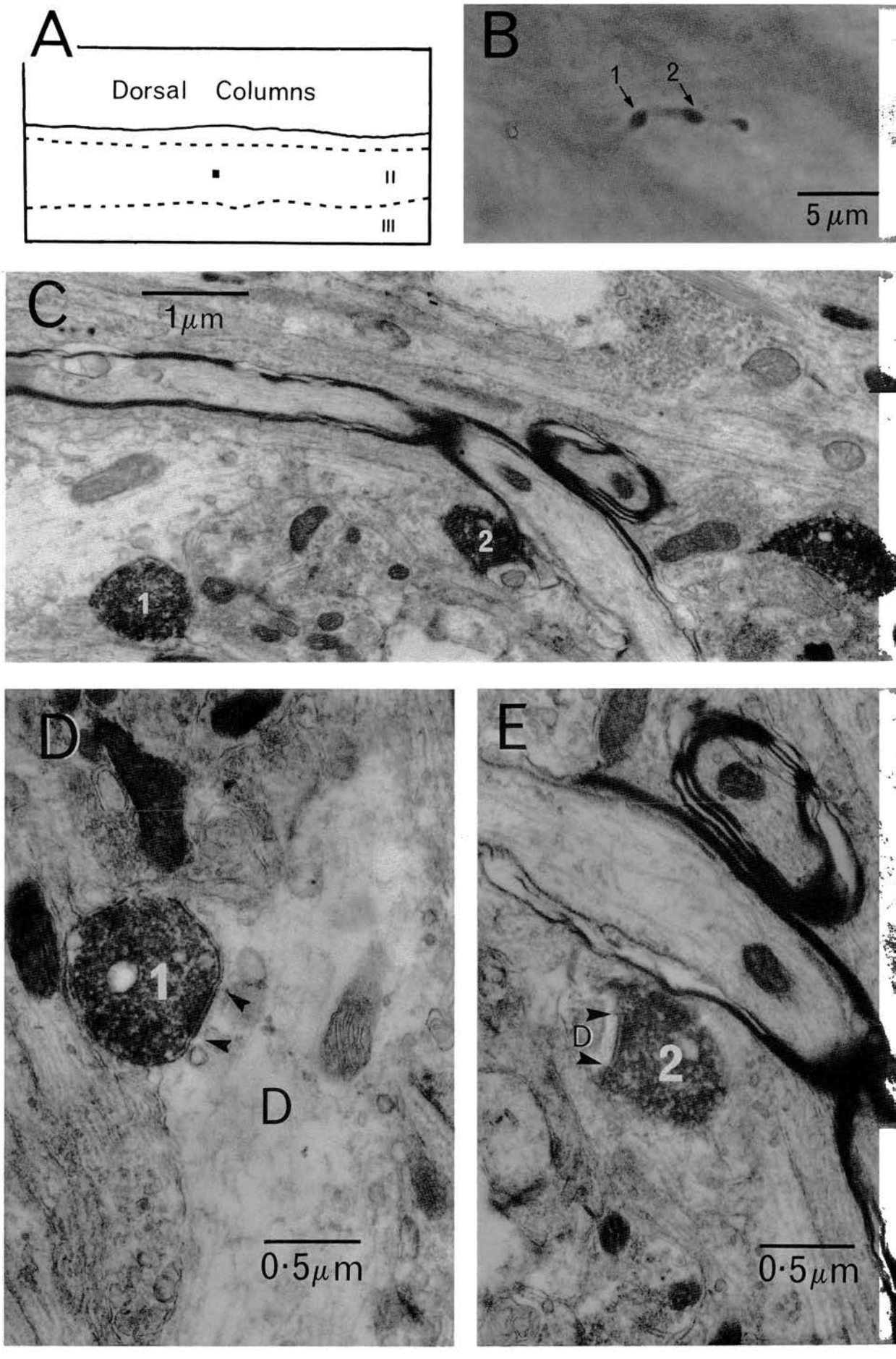

Fig. 7.

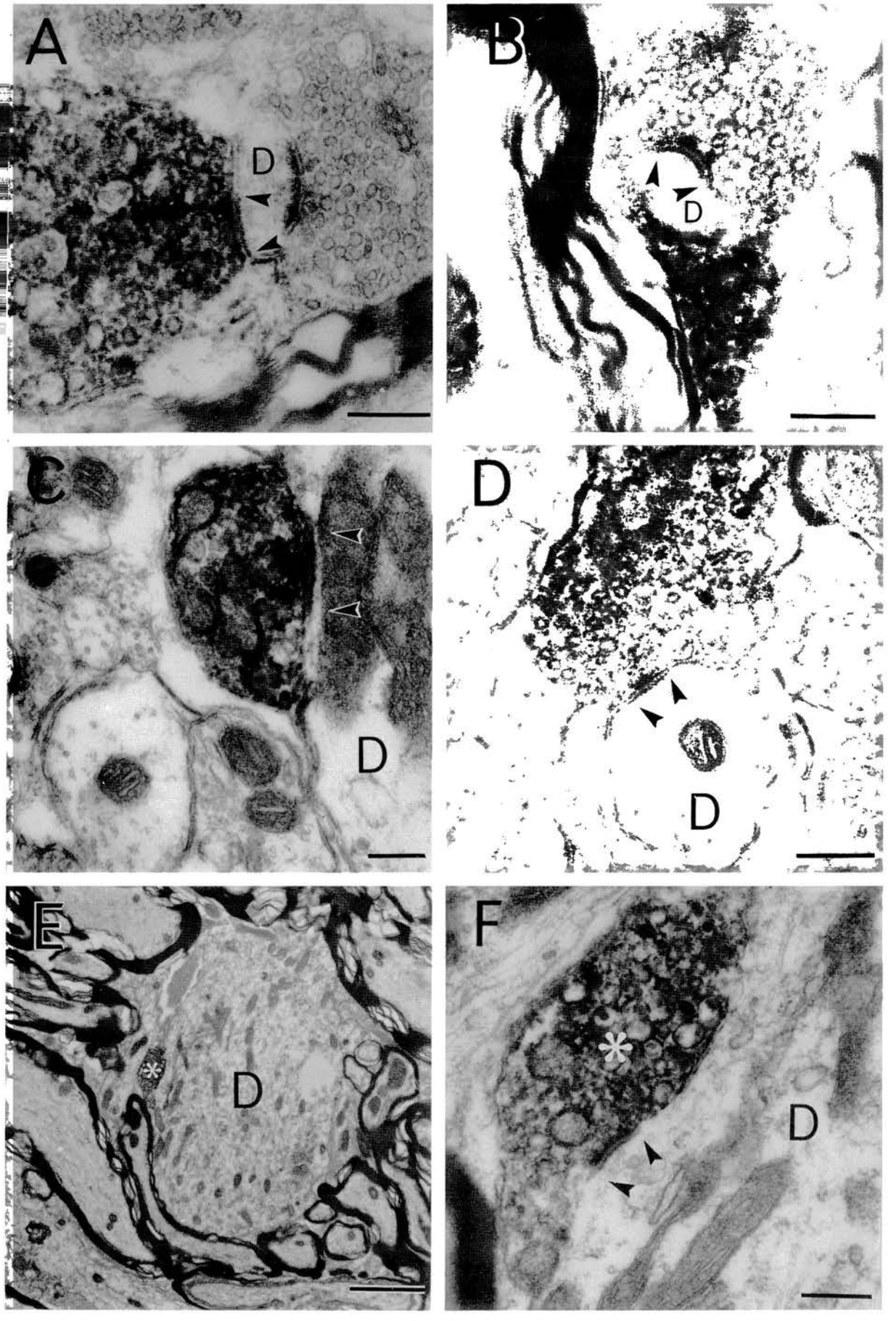

Fig. 8.

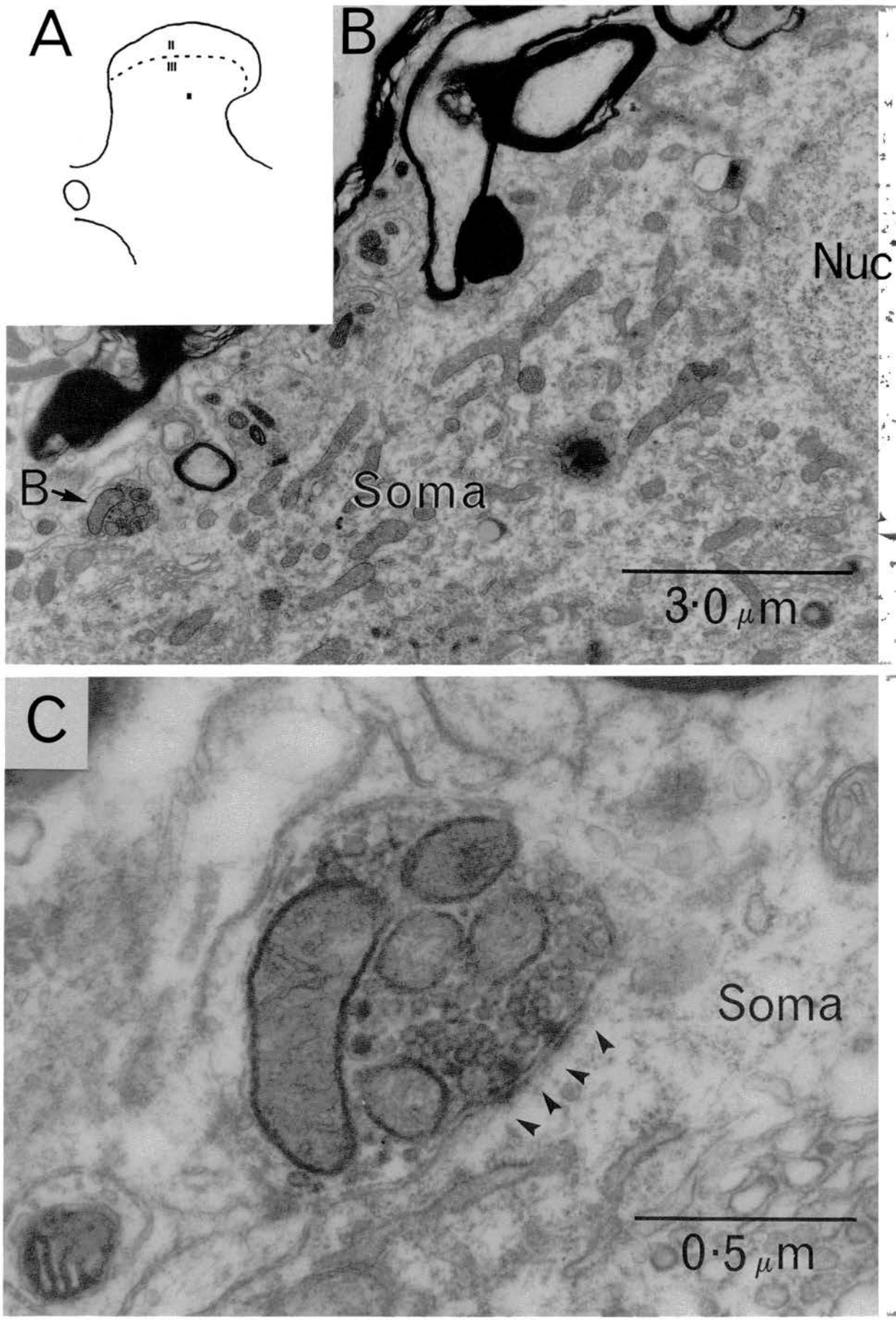

Fig. 9.

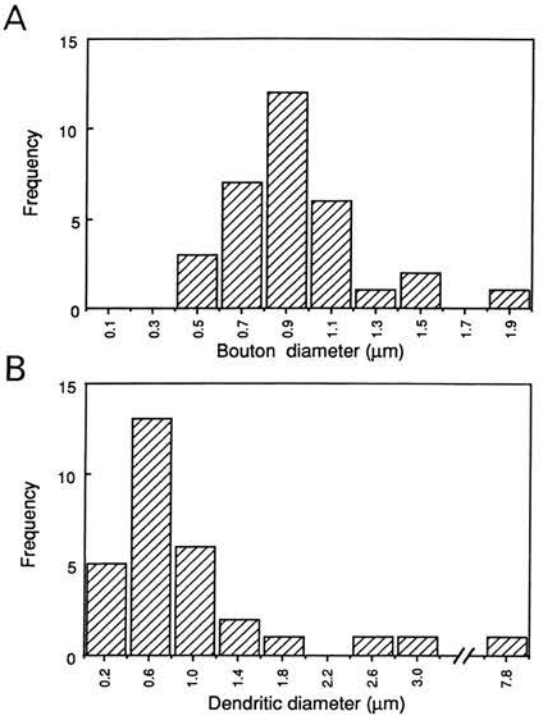

Fig. 10. (A) Frequency distribution histogram of immunolabelled bouton diameters. The majority (85%) of profiles fall within the range 0.6–1.2 μm (mean diameter = 0.94 μm). (B) Frequency distribution histogram of postsynaptic dendritic diameters. Immunolabelled boutons synapse most commonly with medium-sized (0.5–2.0 μm diameter) dendrites. Large calibre dendritic shafts (>2.0 μm diameter) also receive input from TH-immunoreactive axonal endings, as do very small (<0.5 μm diameter) dendrites.

in the lumbosacral region belong to descending systems, since we did not observe any catecholamine-containing neuronal perikarya in this region. This is consistent with previous studies in the cat.[56] In the rat, catecholaminergic perikarya have been demonstrated in the upper sacral cord whose axons project locally and contribute to the catecholaminergic fibre network of this region.[23,67]

It is possible that a small number of dopamine-containing terminals in the dorsal horn may be derived from sensory neurons. Price and Mudge[71] have reported that a small population of adult rat dorsal root ganglion cells are immunoreactive for TH, but not dopamine-β-hydroxylase. However, due to the paucity of these cells, they are unlikely to make a significant contribution to the spinal cord catecholamine content.

Distribution and orientation of tyrosine hydroxylase-immunoreactive fibres

The present study was restricted to the first four laminae of the spinal dorsal horn. Throughout this region large numbers of axons immunoreactive for TH can be localized, laminae I, II and IV possessing a denser innervation than lamina III.

Within the superficial laminae (laminae I and II), the axons exhibit a prominent rostrocaudal orientation, with the highly varicose fibres travelling for considerable distances without branching. This pattern of descent for fibres in these layers has been described previously.[11,19,32] The dendritic arbors of neurons in this region are orientated in a similar way (see Ref. 93 for review), and it seems reasonable to suggest that the TH-immunoreactive fibres form synaptic contacts with these parallel-arranged dendrites.

Sugiura *et al.*[87] have shown using a *Phaseolus vulgaris*-leucoagglutinin intracellular staining technique in the guinea-pig that cutaneous unmyelinated (C) fibres within lamina I distribute as long, rostrocaudally orientated strands, with relatively few branches. Similarly, Gobel *et al.*[37] observed that ultrafine varicose axons within lamina I, labelled following application of horseradish peroxidase (HRP) to cut dorsal roots, travel for considerable distances in the rostrocaudal direction without branching. It has been suggested that the similar orientation of catecholaminergic axons and fine primary afferent axons within lamina I could imply a close association between the two groups. However, our ultrastructural observations did not reveal immunolabelled boutons synapsing with other axon terminals.

Within laminae III and IV, the orientation of the immunolabelled axons changes from a rostrocaudal to a dorsoventral orientation. Many laminae III and IV cells possess dorsally directed dendrites, which extend upwards, penetrating laminae I–III (see Ref. 93 for review). The orientation of the immunolabelled axons therefore appears to change to accommodate the different arborization pattern formed by the cells in this region. In this way, the catecholamine-containing varicose fibres may remain parallel to their target dendrites.

TH-immunoreactive axons were not uniformly distributed across the mediolateral extent of the dorsal horn. In a similar way to the distribution of corticospinal axons,[15] the catecholamine-containing fibres were predominantly located in the medial part of the horn. This heterogeneous arrangement of the dorsal horn in the mediolateral direction may help us to appreciate the interesting findings of Swett and Woolf[88] and Molander and Grant.[66] These authors mapped the distribution of hind-limb nerves in the rat, and found that the tibial nerve that innervates the glabrous skin on the hind-foot projects to the medial third of the horn. Furthermore, Réthelyi *et al.*[74] have observed differences in axonal sprouting between medial and lateral sectors following neonatal capsaicin treatment. All of these findings suggest a unique structure for the medial sector of the spinal dorsal horn onto which selective primary afferent, corticospinal, and catecholamine-containing axons all terminate.

Ultrastructural observations

Our observations with the electron microscope demonstrate that the descending catecholaminergic

projections to laminae I–IV of the cat spinal dorsal horn form symmetrical (Gray type II) synaptic specializations with their postsynaptic targets.

The majority of immunolabelled boutons (95%) was found in association with dendrites, while the remainder (5%) were seen in contact with neuronal somata. On no occasion were axo-axonic synapses observed. We cannot state conclusively that catecholamine-containing boutons do not contact other axon terminals, but if this type of synaptic arrangement does exist in the cat lumbosacral spinal cord then it is extremely rare. Hagihira *et al.*[39] also reached this conclusion in the rat using a similar technique. The majority of axodendritic synapses was found on medium-sized dendrites but contacts upon large calibre dendritic shafts and small diameter dendrites were not uncommon. It appears, therefore, that contacts are found throughout the dendritic arbors of dorsal horn cells.

Boutons immunolabelled for TH were found to contain two distinct types of vesicle: (i) small, irregularly shaped, agranular vesicles, and (ii) prominent dense-core vesicles. The small agranular vesicles are likely to contain catecholamine since they are rendered electron dense by permanganate fixation.[79] This type of precipitation reaction is selective for monoaminergic neurotransmitters.[43,44] Dense-core vesicles can be demonstrated in axon terminals containing transmitters other than catecholamines.[44] Recent evidence indicates that some of these vesicles contain neuropeptides,[64] and a number of studies have demonstrated the co-existence of catecholamines with neuropeptides in the central nervous system.[14,26,63,95] The locus coeruleus, A1/C1, A2/C2 and A4 noradrenergic cell groups have been shown to be immunoreactive for neuropeptide Y,[26,95] and, in addition, the majority of A6 profiles are also positive for galanin[63] and enkephalin.[14] Therefore, the dense-core vesicles observed within TH-immunoreactive boutons may contain some or all of these peptides. The functional significance of co-existence between catecholamines and neuropeptides will be discussed below.

The findings reported in this study are in contrast to those described by Descarries *et al.*[22] and Seguéla *et al.*[81] for ascending catecholaminergic systems in the rat cerebral cortex. These authors found that the majority of catecholamine-containing terminals (80–95%) lacked the membrane specializations of typical synaptic contacts. In this respect, it was proposed that the ascending projections exert a diffuse effect on postsynaptic structures. In contrast, we propose that the catecholamine-containing terminals in the spinal cord of the cat are organized with a high degree of specificity. An earlier report by Satoh *et al.*[79] concluded that most noradrenergic terminals within the substantia gelatinosa (lamina II) of the rat did not form identifiable synaptic junctions. However, the potassium permanganate fixation technique used by these authors results in poor ultrastructural preservation and is not selective for the noradrenergic terminals. Hökfelt *et al.*[43,44] have shown that a number of monoamines can reduce permanganate to manganese dioxide, the strongest reaction of all being achieved with serotonin (5-HT). Since 5-HT is two to six times more abundant within the spinal cord than noradrenaline (NA),[2,98] and immunocytochemical studies have shown a dense network of serotoninergic axons to be present within the dorsal horn,[76] the majority of boutons observed by Satoh *et al.*[79] could have been serotoninergic. Consistent with this idea, Maxwell *et al.*[62] reported that many 5-HT-immunoreactive axons within the substantia gelatinosa in the rat lack the membrane specializations of typical synaptic contacts. Studies using autoradiography[75] and immunocytochemistry[1,39] have shown that most catecholaminergic terminals within the spinal cord do form typical synaptic contacts. The approach used by Ruda *et al.*[75] was based upon the ability of neurons which use NA as a transmitter to take up tritiated NA [^{3}H]NA at their axonal endings. In the rat trigeminal dorsal horn, many labelled axons were found which formed asymmetrical (Gray type I) synapses. Although the autoradiographic approach used by these authors[75] probably resulted in accumulation of [^{3}H]NA within noradrenergic processes it has been shown that a nonspecific uptake by other terminals is possible. There is good evidence that dopamine-containing neurons can accumulate [^{3}H]NA[21,33,69,73,85] and under particular conditions an uptake of [^{3}H]NA might also take place with serotoninergic nerve cells.[33,34] On this basis, uptake studies may not allow autoradiographic distinction of noradrenergic nerve endings. Immunocytochemical studies in the rat dorsal horn have also demonstrated that noradrenergic terminals exhibit membrane specializations of typical synaptic junctions.[1,39] Junctions were mostly,[1] or exclusively,[39] asymmetrical (Gray type I). In the present study we report that contacts made by catecholaminergic boutons in the cat dorsal horn are symmetrical (Gray type II), thus indicating that differences may exist between species in the morphology of synaptic specializations formed by catecholamine-containing boutons. Significantly, the patterns of termination and the postsynaptic targets are similar in both species.

Functional considerations

A number of groups have investigated the actions of sympathomimetics upon dorsal horn neurons which were activated by peripheral stimulation. In general, the predominant effect is an inhibition of activity,[4,20,25,29,30,41,46,80] although occasional excitation is also seen.[4,46,80] These findings are consistent with the effects of electrically stimulating brainstem nuclei known to contain catecholaminergic cell bodies.[29,35,42,65,77,78,97] On the basis of electrophysiological evidence, it appears that catecholamines exert both presynaptic[13,17,40,49,55,94] and postsynaptic[4,25,48,68] actions in regulating sensory transmission through the

dorsal horn. In our present study we did not find any examples of immunolabelled boutons forming synaptic junctions with other axon profiles and conclude that in the cat, catecholaminergic axons do not directly act upon primary afferent terminals to produce presynaptic inhibition. Nevertheless, presynaptic actions may be exerted through an inhibitory local circuit neuron. Wohlberg *et al.*,[94] using an isolated frog spinal cord preparation, obtained evidence that NA acts indirectly to produce hyperpolarization of primary afferent terminals. When synaptic transmission was blocked by application of Mn^{2+}, Mg^{2+}, procaine or tetrodotoxin, the hyperpolarization to NA was subsequently reduced or abolished. Furthermore, mephensin, a reported blocker of polysynaptic pathways, also attenuated with hyperpolarization evoked by NA.[94]

Ultrastructural analysis confirms that TH-immunoreactive boutons form synaptic contacts with the dendrites and somata of cells throughout laminae I–IV. These observations favour the idea of a postsynaptic mechanism of action for catecholaminergic axons within the spinal dorsal horn. Furthermore, our analysis of TH-immunoreactive boutons in the spinal dorsal horn suggests that catecholamines may regulate the responses of a number of different neuronal cell types. Lamina I contains projection neurons of the spinothalamic tract[12] in addition to local circuit neurons.[5] The high density of catecholaminergic fibres in this region could depress activity of thalamic projection neurons as well as inhibit local circuit neurons. Lamina II of the dorsal horn is composed almost entirely of local circuit neurons. The two major types of cell are the stalked cells and the islet cells (see Ref. 93 for review), each of which could receive contacts from catecholaminergic boutons. The neuropil of the superficial dorsal horn also contains dendrites of neurons with cell bodies in deeper lamina of the dorsal horn, such as laminae III–V (see Ref. 93 for review). Therefore it is possible that distal dendrites of these cells receive catecholaminergic input via laminae I and II. Lamina IV contains many projection neurons of the spinocervical tract and postsynaptic dorsal column system (see Ref. 9 for review). An inhibitory action by catecholamines upon these cells would modify spinal input to the lateral cervical nucleus and the dorsal column nuclei.

A number of authors have reported that ionophoretically applied catecholamines depress nociceptive responses with greater efficacy than responses to innocuous stimuli,[4,20,29,30,41] and it has been proposed that the descending catecholaminergic neurons selectively depress activity generated by noxious stimuli. Other authors, however, have failed to demonstrate any selectivity.[46,80] The postsynaptic target neurons of TH-labelled axons were not identified in the present study. However, on the basis of the primary afferent inputs to the different laminae of the dorsal horn, several inferences may be drawn. The high density of TH-positive boutons within laminae I and II, where high threshold Aδ and C fibres terminate,[7,57,87] infers that a depression of nociceptive transmission may occur in this region. Furthermore, in laminae III and IV, where A$\alpha\beta$ fibres terminate (see Refs 9, 10 for review), the catecholaminergic axons may depress transmission of innocuous input. Until the precise nature of the postsynaptic targets of catecholaminergic fibres is resolved, interpreting the effects observed during NA ionophoresis will remain difficult. This would include knowing whether catecholamine-containing axodendritic synapses on distal dendrites are adjacent to excitatory synapses conveying nociceptive information. A selective inhibition of nociceptive pathways may be achieved by such a local circuit through shunting inhibition.

Brainstem stimulation studies indicate that a selective inhibition of nociceptive pathways can be achieved through activation of the locus coeruleus[42,65,97] and dopaminergic A11 group.[29] The subcoeruleus–parabrachial nuclei[35] and Kölliker–Fuse nucleus[97] produce non-selective inhibition of sensory transmission. An intriguing finding to emerge from these studies is the insensitivity of coerulospinal inhibition to reversal by adrenergic antagonists.[42,97] Hodge *et al.*[42] found that inhibition from the locus coeruleus was unaltered by depletion of brain and spinal cord NA with reserpine (1–2 mg/kg, i.p.). Furthermore, Zhao and Duggan[97] found that while idazoxan antagonized NA-induced inhibition, it failed to block the inhibition of dorsal horn neurons induced by stimulation of the locus coeruleus or Kölliker–Fuse nucleus. One possible explanation of these results is that other transmitters are co-released with NA, and depletion of NA alone is insufficient to reduce a postsynaptic action. It has been discussed earlier that the dense-core vesicles observed within TH-immunoreactive boutons are likely storage sites for candidate transmitter substances such as enkephalin, galanin or neuropeptide Y. It is significant, therefore, that a co-release of NA with neuropeptide Y has been reported in the peripheral nervous system,[86] and galanin has been shown to depress nociceptive reflexes in the isolated spinal cord–tail preparation of the newborn rat.[96]

CONCLUSION

Our analysis of TH-immunoreactive axons in laminae I–IV of the spinal dorsal horn of the cat indicates that boutons form symmetrical (Gray type II) synaptic specializations upon dorsal horn neurons, but not other axon terminals. This favours the idea of a postsynaptic action of catecholamines to regulate sensory transmission through the dorsal horn.

Acknowledgements—Special thanks are due to Mrs H. Anderson and Mr C. Warwick for excellent technical assistance. We are indebted to Prof. A. G. Brown, Prof. A. W. Duggan and Dr C. I. Ingham for critically reviewing the manuscript. The study was supported by the Medical Research Council.

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(*Accepted* 16 *April* 1991)

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ADONIS 000689939124909G

BRES 24909

Ultrastructural analysis of noradrenergic nerve terminals in the cat lumbosacral spinal dorsal horn: a dopamine-β-hydroxylase immunocytochemical study

Christopher A. Doyle and David J. Maxwell

Department of Preclinical Veterinary Sciences, University of Edinburgh, Edinburgh (U.K.)

(Accepted 30 July 1991)

Key words: Cat; Dopamine-β-hydroxylase; Immunocytochemistry; Noradrenaline; Spinal cord; Ultrastructure

Noradrenaline-containing nerve terminals within the cat spinal dorsal horn were studied by immunocytochemical localization of dopamine-β-hydroxylase. Immunoreactive terminals formed symmetrical (Gray type II) synaptic specializations with dendrites and somata throughout laminae I–IV, but no junctions were formed with other axons. These findings suggest that noradrenaline regulates sensory transmission through the dorsal horn via a postsynaptic action.

Noradrenaline (NA) containing axons and terminals in the dorsal horn of the spinal cord of mammals[3,14,18,21], are derived from the nucleus locus coeruleus, nucleus subcoeruleus and other pontine NA cell groups[15,21,22]. These regions correspond to the A5–A7 cell groups originally described by Dahlström and Fuxe[2]. Ionophoresis of NA[6,9,10] and stimulation of pontine NA cell groups[15,22] has been shown to depress sensory transmission through the spinal dorsal horn, an effect which may be selective for activity generated by noxious stimuli[6,9,15,22]. Results from electrophysiological studies suggest that NA regulates spinal sensory transmission through both a pre-synaptic action upon primary afferent terminals[1,13] and a post-synaptic[12,16] action upon dorsal horn neurons. However, ultrastructural evidence from immunocytochemical studies favours a post-synaptic action[5,8]. In a previous study we used an antiserum against tyrosine hydroxylase (TH) to label presumed catecholamine (CA) containing axons in the cat spinal dorsal horn[5]. Since TH is the first enzyme in the CA biosynthetic pathway, an immunocytochemical analysis of NA axons may be complicated by labelling of dopaminergic fibres. Owing to this difficulty, and the recent discovery of neurons in the brain that contain TH but not CA[20], we have re-examined the ultrastructural features of NA axons in the dorsal horn using an antiserum to dopamine-β-hydroxylase (DBH), the enzyme which converts dopamine to NA.

Three female cats were anaesthetized with sodium pentobarbitone (40 mg/kg) and fixed by transcardial perfusion as described previously[5]. The lumbosacral (L_7–S_1) spinal cord was dissected out and 40 μm transverse and sagittal sections were cut on a Vibratome. The antiserum to DBH was purchased from Eugene Tech (New Jersey), and diluted 1:1000 in phosphate buffered saline. Tissue sections were incubated overnight in this primary antiserum supplemented with 1% donkey serum, 0.05% sodium azide and 0.3% Triton X-100. Although ultrastructural preservation is compromised by the addition of Triton X-100 in the incubation medium, its inclusion is necessary for successful DBH immunocytochemical staining. The presence of DBH was visualized using the avidin-biotin-peroxidase complex (ABC) technique[11] using 3,3-diaminobenzidine as a chromogen. The sections were then processed for electron microscopy as previously described[5].

Light microscopic inspection revealed large numbers of DBH-immunoreactive axons throughout the first 4 dorsal horn laminae. The distribution and patterns of termination of these structures was similar to that reported previously for fibres immunoreactive for TH[5], with laminae I, II and IV possessing a greater innervation than lamina III. This distribution has also been described in the caudal spinal cord of rat[18]. Analysis of the superficial dorsal horn (laminae I and II) revealed an extensive plexus of very long, varicose, rostrocaudally-orientated DBH axons. Lamina II also contained a number of shorter axons extending in a dorsoventral direction. In laminae III and IV, DBH fibres were short with

Correspondence: C.A. Doyle, Department of Preclinical Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh, EH9 1QH, U.K.

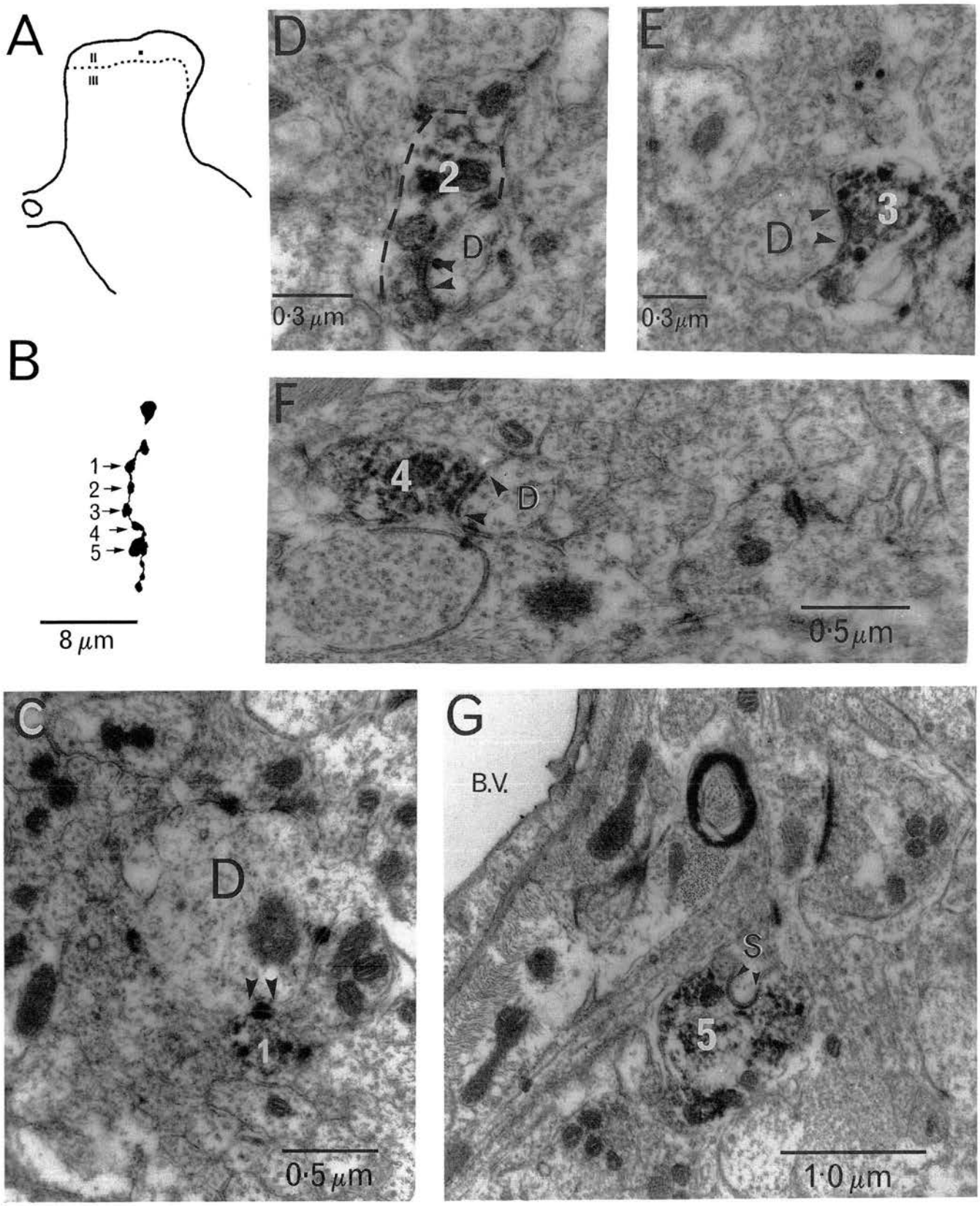

Fig. 1. Correlated light and electron microscopic analysis of a dopamine-β-hydroxylase (DBH)-immunoreactive axon (Triton X-100 treated material). A: position of the axon in lamina II of the dorsal horn (■). B: light microscopic appearance of the axon. Boutons 1–5 are illustrated in the electron micrographs (C–G). These boutons formed symmetrical (Gray type II[7]) axo-dendritic synaptic junctions. Active zones (between the arrowheads) of boutons 2–5 and their post-synaptic dendrites (D) are shown in D–G. Bouton 1 additionally formed a punctum adhaerens (arrowheads) with a dendrite, D, (C). Synaptic contacts were observed upon small (< 0.5 μm diameter: D and F) and medium-sized (0.5–2.0 μm diameter: E) dendrites, as well as dendritic spines (G). In G; B.V., blood vessel.

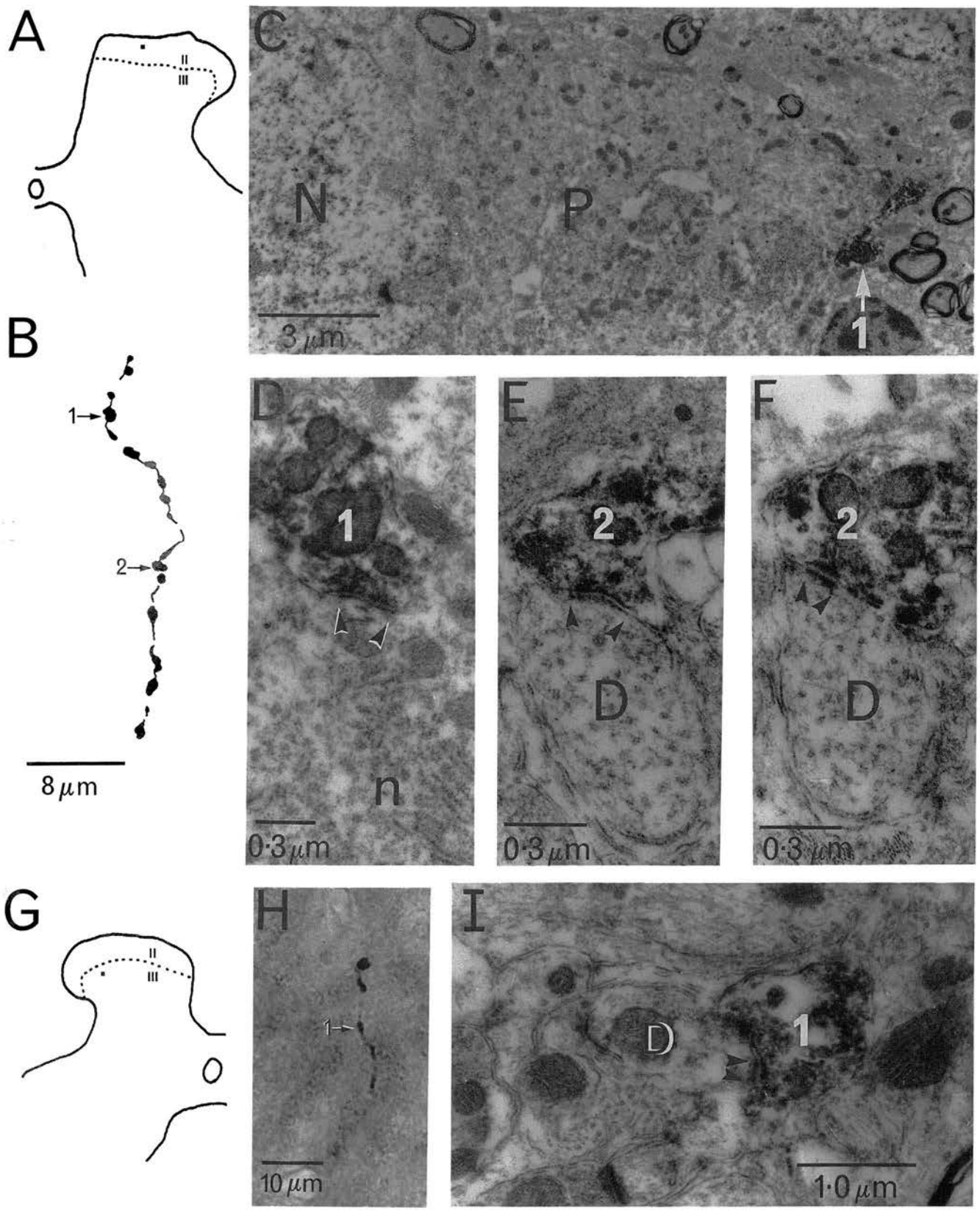

Fig. 2. Correlated light and electron microscopic analysis of two further DBH-immunoreactive axons (Triton X-100 treated material). The first fibre was located at the lamina I–II border (■) (A), and its light microscopic appearance is shown in (B). Boutons 1 and 2 are illustrated in electron micrographs C–F. C: a low power electron micrograph showing close appostition between bouton 1 and a perikaryon (P), nucleus, N. D: at higher power a symmetrical synaptic junction is revealed (between the arrowheads), n, Nissl substance. E, F: serial sections of an axo-dendritic synapse formed by bouton 2. The morphology of this junction changed as it was followed through serial sections: in micrograph E it appears symmetrical, whereas in F it is asymmetrical. G: the position of a DBH-fibre in lamina III (■). H: light micrograph of the immunocytochemically stained axon. The varicosity marked (1) is shown in an electron micrograph I. This bouton forms a symmetrical synaptic junction (between the arrowheads) with a small dendrite (D).

few 'en passant' varicosities. Most axons were organized dorsoventrally, but axons running in both the mediolateral and rostrocaudal planes were also seen.

Correlated ultrastructural analysis confirmed that these varicosities were synaptic boutons. Tissue preservation at the electron microscope level was not optimum due to treatment with Triton X-100. However, sufficient detail was retained to examine synaptic organization. In all, 23 boutons exhibiting DBH-immunoreactivity were examined through serial sections with the electron microscope. NA-containing boutons usually formed single synapses on dendrites (95% of synapses) or somata (5% of synapses). Two boutons, however, formed synapses with more than one post-synaptic target. DBH-positive boutons formed synapses most commonly with medium-sized (0.5–1.0 μm diameter) dendrites (Figs. 1E, 2E,F, I), but contacts upon small (< 0.5 μm diameter) dendrites (Figs. 1D,F) and dendritic spines (Fig. 1G) were not uncommon. Immunolabelled boutons were not observed to form axo-axonic arrangements, although they were often found adjacent to vesicle-containing structures. Synaptic junctions formed by these axonal terminals were usually of the symmetrical type (Gray type II[7]), with a slight accumulation of electron dense material on the presynaptic and postsynaptic sides of the junction. One synapse (Fig. 2F) appeared to be Gray type I[7] (asymmetrical), although in a serial section it looked symmetrical (Fig. 2E). Non-synaptic, puncta adhaerentia were also seen (Fig. 1C).

Boutons were characteristically packed with small, irregularly-shaped, agranular vesicles (Figs. 1 and 2) together with several dense core vesicles (Figs. 1D,E). Immunoreactivity appears to be associated with both types of vesicle.

The present study confirms our original observations that CA-containing terminals in the spinal dorsal horn are organized with a high degree of synaptic specificity[5]. This contrasts with the proposed organization of ascending CAergic systems in the rat cerebral cortex[4,19], where the majority of NA-containing terminals (80–95%) lack membrane specializations of typical synaptic contacts. An earlier report by Satoh et al. in the rat[17], concluded that few NA terminals in lamina II of the spinal dorsal horn form synaptic junctions. However, our immunocytochemical studies in the cat[5] and those of Hagihira et al.[8] in the rat have demonstrated that most descending CA-containing terminals form identifiable synaptic junctions. It is interesting to note that junctions in the cat are symmetrical, but those in the rat are asymmetrical[8].

The post-synaptic targets of DBH-immunoreactive axons were very similar to those observed for TH-immunoreactive axons in our previous study[5], i.e. contacts were found upon dorsal horn neurons but not other axons. These findings for the cat have been substantiated in the rat by Hagihira et al.[8], and cumulatively these data strongly suggest that the actions of CA in the spinal dorsal horn operate through a post-synaptic mechanism.

The high degree of accordance found between the present study and our previous analysis of spinal CA's using antibodies to TH[5], strongly suggests that equivalent structures were labelled by each antiserum.

Special thanks are due to Heather Anderson for her skilled technical assistance. We are also grateful to Dr. Cali Ingham for carefully reviewing the manuscript. This study was supported by the Medical Research Council.

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Immunocytochemical evidence for a catecholaminergic innervation of postsynaptic dorsal column neurons in the cat

C.A. Doyle and D.J. Maxwell

Department of Preclinical Veterinary Sciences, University of Edinburgh, Edinburgh EH9 1QH

Catecholamine-containing axons, originating in the brain, descend and terminate in every lamina of the lumbosacral spinal grey matter (Westlund *et al.* 1984). Thus, the potential number of different kinds of targets for catecholaminergic input is large. In the dorsal horn, none of these targets has been identified. Recent work has shown that laminae III and IV contain an appreciable density of axons immunoreactive for tyrosine hydroxylase (Doyle & Maxwell, 1991). Furthermore, laminae III and IV also contain the perikarya and dendritic arbors of somatosensory projection neurons whose axons ascend the dorsal columns (Brown & Fyffe, 1981). In the present study we have examined the possibility that these second order neurons are one of the targets for input from descending catecholamine-containing axons.

Postsynaptic dorsal column neurons were retrogradely labelled with horseradish peroxidase using a pellet-implantation technique (Enevoldson & Gordon, 1989) in the dorsal columns (T10–12) of 4 adult cats anaesthetized with pentobarbitone (40 mg/kg I.P.). After a recovery period of 32–48 hours, the animals were anaesthetized again with pentobarbitone and fixed by transcardial perfusion with a solution containing 4% paraformaldehyde, 0.1% glutamaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.4. Sections of lumbosacral (L6–S1) spinal cord containing labelled neurons were processed immunocytochemically to reveal the presence of tyrosine hydroxylase and dopamine-β-hydroxylase.

One hundred and thirty four retrogradely labelled neurons were examined under high power (×1000) with the light microscope. Thirty three per cent of cells had immunoreactive varicosities closely apposed to their somata and proximal dendrites (mean = 3 varicosities/cell; range = 1–13). Preliminary electron microscopical investigations have confirmed that some of these appositions are regions of synaptic contact.

These findings suggest that catecholamines may regulate sensory transmission through the postsynaptic dorsal column pathway via a postsynaptic action upon its cells of origin.

Supported by the MRC.

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BRES 25537

Neuropeptide Y-immunoreactive terminals form axo-axonic synaptic arrangements in the substantia gelatinosa (lamina II) of the cat spinal dorsal horn

Christopher A. Doyle and David J. Maxwell

Department of Preclinical Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh (UK)

(Accepted 10 November 1992)

Key words: Neuropeptide Y; Immunocytochemistry; Spinal cord; Cat; Ultrastructure; Axo-axonic synapse

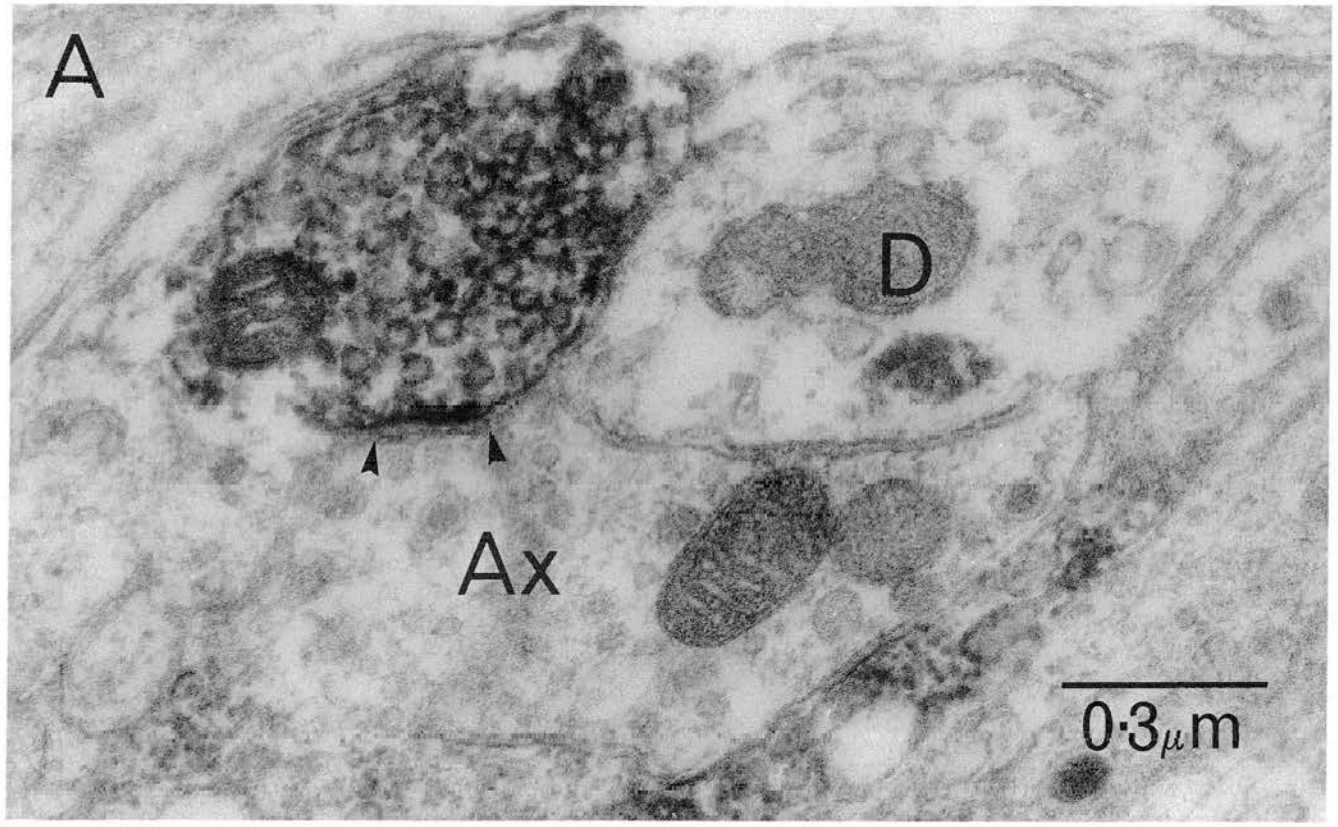
The ultrastructural organization of nerve terminals containing neuropeptide Y-immunoreactivity was studied in the substantia gelatinosa of the cat spinal dorsal horn. Seventy immunoreactive boutons were examined through serial sections and 67 of them were found to form between one and five synaptic junctions with dendrites (59.5% of synapses), somata (3% of synapses) and other axon terminals (37.5% of synapses). The postsynaptic axon terminals were often the central boutons of glomeruli. These findings suggest that neuropeptide Y regulates spinal sensory transmission through both a postsynaptic action upon dorsal horn neurons and a presynaptic action upon primary afferent terminals.

Neuropeptide Y (NPY) has been shown by immunocytochemical and radioimmunoassay techniques to be one of the most abundant and widely distributed peptides within the mammalian central nervous system[1,4]. Examination of the spinal cord has revealed that axon terminals immunoreactive for NPY are particularly concentrated within the substantia gelatinosa (layer II) of the dorsal horn in a wide range of species[3,7,12,16]. These fibres probably originate from descending pathways[3,8] and intrinsic spinal neurons[12,16]. NPY is generally thought to be absent from primary sensory neurons[7,18], but a report by Lindh et al.[13] suggests that a few dorsal root ganglion cells in the cat may contain it. Microinjection of NPY into the cat dorsal horn has been shown to reduce the release of substance P that occurs following electrical stimulation of unmyelinated primary afferents[6]. Following an initial period in which substance P release over the whole dorsal horn is reduced, inhibition subsequently becomes restricted to laminae I and II. Furthermore, NPY can reduce the depolarization-evoked release of substance P from dorsal root ganglion cells in culture[17]. In addition, the number of NPY binding sites in the superficial dorsal horn is dramatically reduced when the primary afferent input to this region is removed[10]. These findings suggest that NPY can act presynaptically upon the terminals of primary sensory neurons.

At present, there are no ultrastructural studies of the relationship between NPY-containing axons and other structures in the neuropil of the superficial dorsal horn. The aim of the present study was to examine the fine structural organization of NPY-containing terminals in lamina II of the cat dorsal horn, with particular attention being given to the presence or absence of axo-axonic synaptic junctions.

Two female cats were anaesthetized with sodium pentobarbitone (40 mg/kg) and fixed by transcardial perfusion as previously described[5]. The lumbosacral (L_7–S_1) spinal cord was dissected out and 40 μm transverse and saggital sections were cut on a Vibratome. The antiserum to NPY was purchased from Cambridge Research Biochemicals (Cambridge), and diluted 1:2,000 in phosphate buffered saline (PBS) containing 1% donkey serum and 0.05% sodium azide.

Correspondence: C.A. Doyle, Department of Preclinical Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK. Fax: (44) (31) 6620790.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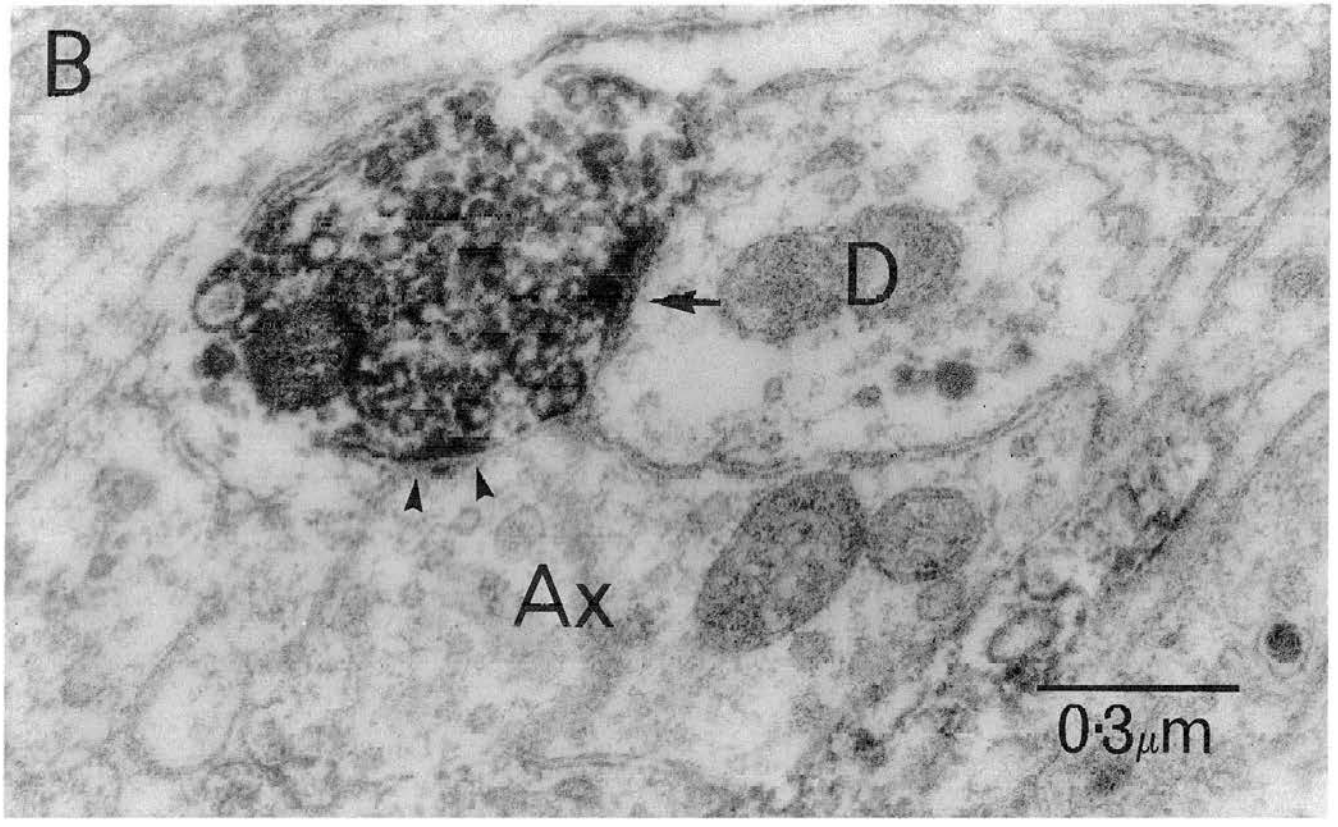
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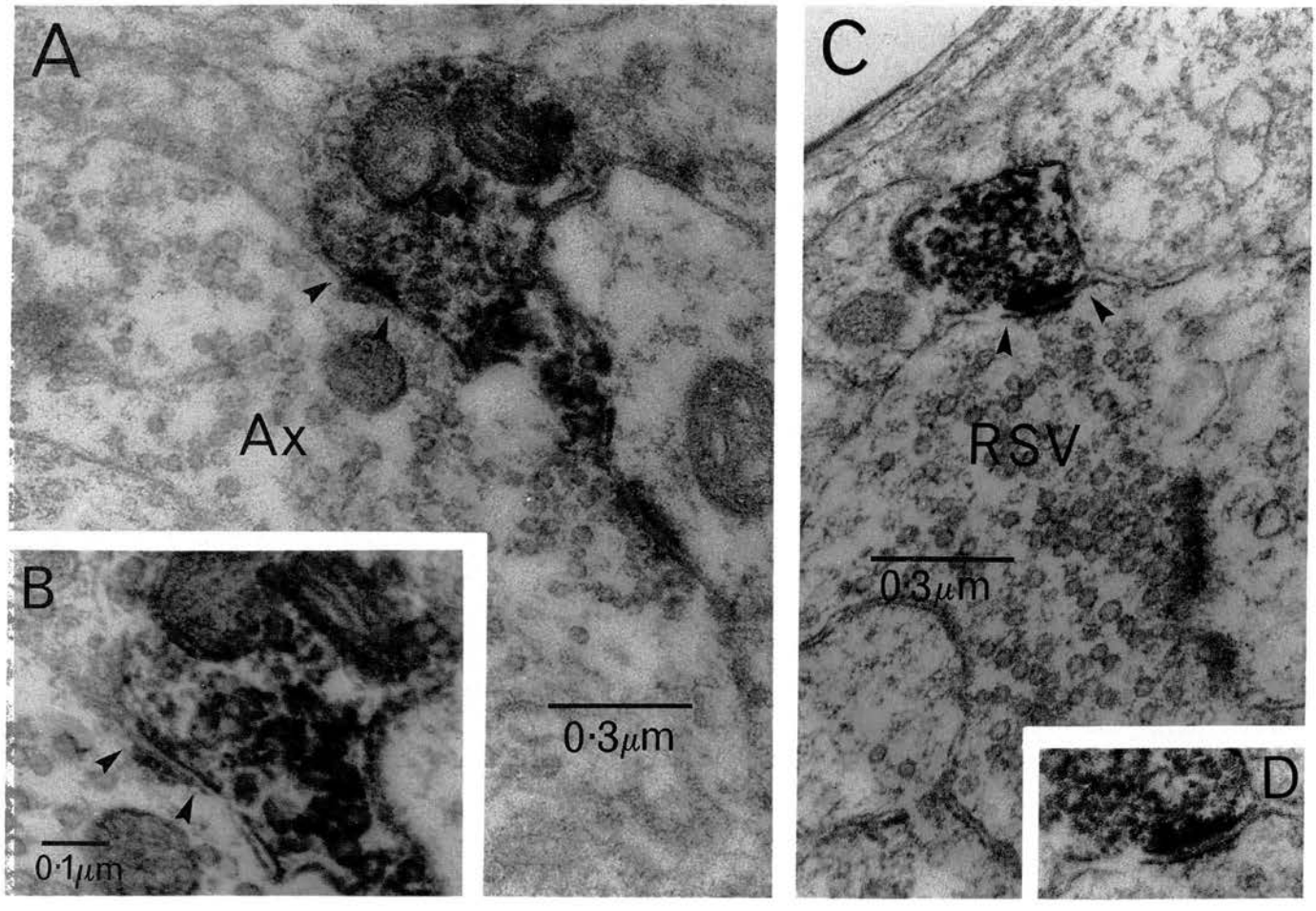

Fig. 2. A shows an NPY-immunoreactive bouton which forms an asymmetrical synaptic junction (between the arrow heads) with a large unlabelled axon terminal (Ax). In the serial section B, the synaptic junction (between the arrow heads) can be defined by the following criteria: (i) a well-defined synaptic cleft; (ii) a marked postsynaptic specialization producing an asymmetrical synaptic junction; and (iii) an accumulation of vesicles adjacent to the presynaptic membrane. C shows an NPY-containing terminal which is presynaptic to a central glomerular bouton which contains spherical vesicles of uniform diameter (RSV[11]). The symmetrical synaptic junction formed between these structures can be seen (between the arrow heads), but it is more clearly resolved at higher magnification (D).

After a 30 min pre-incubation in 10% donkey serum, sections were incubated overnight in the primary antiserum. This primary antiserum was supplemented with 0.3% Triton X-100 in sections that were to be examined by light microscopy only. In control sections, the NPY antiserum was omitted from the overnight incubation medium. The presence of NPY was visualized using the avidin-biotin-peroxidase complex (ABC) technique using 3,3-diaminobenzidine as the chromogen. Sections were then processed for electron microscopy as previously described[5].

Light microscopic inspection revealed large numbers of NPY-immunoreactive terminals in laminae I and II with a second, much smaller cluster, being found in lamina IV. This is in accord with previous studies[3,7,12,16]. NPY-terminals were present predominantly as single, isolated, punctate structures (especially in transverse sections), and axonal strands were only observed occasionally. No immunoreactivity was seen in control tissue which was incubated without the NPY antiserum.

Ultrastructural analysis confirmed that these immunostained varicosities were synaptic boutons. In to-

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Fig. 1. Serial sections of an NPY-immunoreactive bouton which forms an axo-axonic synaptic junction (between the arrow heads) with an unlabelled axon terminal (Ax). The polarity of this synapse is from the NPY-containing terminal onto the unlabelled axon. A shows a section of the bouton which was taken mid-way through the synaptic junction. Note the obvious widening of the synaptic cleft and symmetry of the membrane specializations. B shows the final section of the bouton in which the synaptic junction was present. Note the accumulation of vesicles adjacent to the presynaptic membrane and the marked postsynaptic thickening which gives the junction an asymmetric appearance in this particular section. This NPY-immunostained terminal also forms a synaptic junction with the dendrite D (arrow in Fig. B).

tal, 70 boutons exhibiting NPY-immunoreactivity were examined completely through serial sections with the electron microscope and 67 of them formed synaptic junctions. These junctions were usually symmetrical (Figs. 1A, 2C, D), but axo-axonic associations were often asymmetrical (Fig. 2A, B). The ultrastructural organization of these structures was highly complex, with 40% of terminals forming more than one synapse. Almost 60% of synaptic junctions were found upon dendrites (0.35–3.0 μm diameter; see Fig. 1B), 37.5% were found upon unlabelled axon terminals (Figs. 1A, B, 2A–D) and 3% upon somata. Approximately 10% of the NPY-terminals formed axo-axonic junctions alone. Analysis of NPY axo-axonic associations revealed that NPY-containing terminals were usually presynaptic to the unlabelled terminal (Figs. 1, 2). Post-synaptic terminals to NPY-labelled axons were frequently central boutons of glomeruli (Fig. 2C). NPY boutons were characteristically packed with small, irregularly shaped agranular vesicles (Figs. 1, 2), together with several dense core vesicles. Although immunoreactivity was homogenously scattered throughout the cytoplasm, it was particularly associated with the dense core vesicles.

The present study demonstrates that almost all NPY-containing nerve terminals in lamina II of the cat spinal dorsal horn form synaptic specializations. NPY-positive cells have been observed in the dorsal horn after pretreatment with colchicine[12,16], which could give rise to vesicle-containing dendrites. However, in the present study no NPY-containing cells were observed and all of the NPY-containing structures that were present were probably axon terminals. Many of these boutons formed synaptic junctions with the dendrites and somata of dorsal horn neurons (62.5% of synapses), but a large proportion were presynaptic to other axon terminals (37.5% of synapses), including central glomerular boutons and the appearance of these synaptic junctions was similar to that of axo-axonic synapses previously described in the spinal cord[2,14]. Almost all central glomerular boutons degenerate following dorsal rhizotomy[11], thereby demonstrating that they are primary afferent in origin. Hence, the present study suggests that NPY-containing axons in lamina II may regulate spinal sensory transmission through a presynaptic action upon primary afferent terminals in addition to a post-synaptic action upon dorsal horn neurons. Moreover, the finding of NPY-containing boutons in presynaptic apposition to central glomerular boutons, many of which contain substance P[15], may help explain the observation that when NPY is injected into lamina II it reduces the evoked release of substance P from C-fibres[6]. Thus, these axo-axonic arrangements may provide a morphological basis for the analgesia produced when NPY is administered intrathecally[9].

The authors gratefully acknowledge the expert technical assistance of Heather Anderson. The study was supported by the Medical Research Council.

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THE JOURNAL OF COMPARATIVE NEUROLOGY 331:434–444 (1993)

Direct Catecholaminergic Innervation of Spinal Dorsal Horn Neurons With Axons Ascending the Dorsal Columns in Cat

CHRISTOPHER A. DOYLE AND DAVID J. MAXWELL
Department of Preclinical Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, United Kingdom

ABSTRACT

Previous ultrastructural studies have shown that catecholamine-containing nerve terminals in the spinal dorsal horn form synaptic junctions with dendrites and somata, but the identity of the neurons giving rise to these structures is largely unknown. In this study we have investigated the possibility that spinomedullary neurons, which project through the dorsal columns to the dorsal column nuclei, are synaptic targets for descending catecholaminergic axons.

Neurons with axons ascending the dorsal columns were retrogradely labelled after uptake of horseradish peroxidase by their severed axons in the thoracic (T10–T12) or cervical (C2–C3) dorsal columns. After the retrogradely labelled neurons were visualized, the tissue was immunocytochemically stained with antisera raised against tyrosine hydroxylase or dopamine-β-hydroxylase.

Three hundred forty-three retrogradely labelled neurons within laminae III–V of the lumbosacral dorsal horn were examined under high power with the light microscope. In Triton X-100 treated material, over 60% of cells were found to have dopamine-β-hydroxylase-immunoreactive varicosities closely apposed to their somata and proximal dendrites. The number of contacts per cell varied from 1 to 22, with a mean number of 4.5. Fewer cells (34%) received contacts from axons immunoreactive for tyrosine hydroxylase as a consequence of the weaker immunoreaction produced by this antiserum. Correlated light and electron microscopic analysis confirmed that many of these contacts were regions of synaptic specialization and that immunostained boutons contained pleomorphic (round to oval) agranular vesicles together with several dense core vesicles.

These observations suggest that catecholamines regulate sensory transmission through this spinomedullary pathway by a direct postsynaptic action upon its cells of origin. Such an action would be predicted to suppress transmission generally through this pathway.

Key words: descending inhibition, HRP retrograde-transport, immunocytochemistry, somatosensory system, ultrastructure

Catecholamine (CA)-containing nerve terminals have been shown to innervate the spinal gray matter of a number of species including cat (Doyle and Maxwell, '91a,b; Lackner, '80), rat (Fritschy and Grzanna, '90; Schroder and Skagerberg, '85), opossum (Pindzola et al., '88), and primates (Westlund et al., '84). Retrograde tracing techniques have demonstrated that these terminals arise from neurons whose cell bodies are located in supraspinal sites (Björklund and Skagerberg, '79; Blessing et al., '81; Fritschy and Grzanna, '90; Hökfelt et al., '79; Skagerberg et al., '82; Skagerberg and Lindvall, '85; Stevens et al., '82; Westlund et al., '81, '83, '84). The pontine locus coeruleus (A6) and subcoeruleus (A7), Kölliker-Fuse nucleus (A7), and A5 and A4 groups all send descending noradrenergic projections to the dorsal horn, while dopamine-containing terminals are derived from the diencephalic A11 cell group.

Recent ultrastructural studies have demonstrated that CA-immunoreactive axons form synaptic junctions with dendrites and somata of dorsal horn neurons (Doyle and Maxwell, '91a,b; Hagihira et al., '90). Westlund et al. ('90) have shown contacts between these terminals and spinothalamic tract neurons, but at present the identity of most of the neurons contacted by CA-containing terminals is unknown. Laminae III–V of the dorsal horn contain an appreciable density of CA-containing axonal varicosities (Doyle and Maxwell, '91a; Fritschy and Grzanna, '90;

Accepted January 5, 1993.

Lackner, '80; Pindzola et al., '88; Schroder and Skagerberg, '85), in addition to the perikarya and dendritic arbors of somatosensory neurons with axons that project through the dorsal columns to the dorsal column nuclei (postsynaptic dorsal column [PSDC] neurons) (Angaut-Petit, '75a,b; Bennett et al., '83, '84; Brown et al., '83; Brown and Fyffe, '81; Enevoldson and Gordon, '89a; Giesler and Cliffer, '85; Giesler et al., '84; Rustioni, '73, '74, '76; Rustioni and Kaufman, '77; Uddenberg, '68). Hence, there is opportunity for interaction between these two systems. Furthermore, noradrenaline can alter the cutaneous response characteristics of PSDC neurons when ionophoresed into the dorsal horn (Fleetwood-Walker et al., '85), and electrophysiological studies have also shown that PSDC neurons are regulated by descending systems from the brain (Angaut-Petit, '75b; Noble and Riddell, '89).

It therefore follows that PSDC neurons might be one of the targets of descending CA-containing axons. Consequently, we have retrogradely labelled neurons in the dorsal column pathway by means of a horseradish peroxidase (HRP) pellet implantation technique (Enevoldson et al., '84) and immunocytochemically stained CA-containing axons by using antisera raised against tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH). TH is the enzyme that converts tyrosine to dihydroxyphenylalanine (DOPA) and is thus the first enzyme in the CA biosynthetic pathway. Therefore, it is an endogenous protein of dopamine-, noradrenaline-, and adrenaline-containing neurons. DBH catalyses the conversion of dopamine to noradrenaline and is thus present only within noradrenergic and adrenergic neurons. Putative synaptic contacts between labelled cells and immunostained terminals within laminae III–V were analysed by combined light and electron microscopy. A preliminary abstract of this work has been published (Doyle and Maxwell, '92). Nishikawa et al. ('83), using a similar method, have demonstrated serotoninergic synaptic contacts upon PSDC neurons.

MATERIALS AND METHODS

Four adult cats (2.4–3.9 kg) of either sex were used in this series of experiments.

Retrograde labelling

Postsynaptic dorsal column neurons were retrogradely labelled according to the method described by Enevoldson et al. ('84). Cats were deeply anaesthetized with sodium pentobarbitone (40 mg/kg, i.p.) and a dorsal laminectomy was performed at T10–T12 (3 animals) to expose the spinal cord. Strict aseptic conditions were maintained throughout this procedure and normal body temperature was maintained by an electric blanket controlled by a rectal probe. An incision was made in the dura mater and excess cerebrospinal fluid was removed by suction. The spinal cord was kept dry by using tissue paper wicks. Small bilateral lesions were made in the midline region of the dorsal columns with the aid of a razor blade knife, and one or two small agar-HRP pellets were placed into the lesion with dry watchmaker's forceps. When the pellet was in position, the lesion was plugged with small pieces of Sterispon (Allen and Hanbury, Ltd., UK) and a drop of quicksetting cyanoacrylate glue (Loctite, UK) was used to seal the implant site. At the conclusion of surgery animals were given intramuscular injections of penicillin (100,000 units) and allowed to recover from the anaesthetic.

In one cat, a unilateral HRP implant was made in the dorsal columns of the rostral cervical cord (C2–C3). A lesion was then made in the ipsilateral dorsolateral funiculus (DLF) one segment caudal to the implant site to ensure that neurons projecting to the lateral cervical nucleus with axons in the DLF were not transporting HRP.

Fixation of tissue

The animals were allowed to survive for 36–72 hours (T10–T12 implants) or 96 hours (C2–C3 implant). They were then deeply anaesthetized with pentobarbitone (40 mg/kg, i.p.) and transcardially perfused with warm saline (37°C) containing 100 U/ml heparin and 0.1% sodium nitrite. The solution was delivered at a pressure of 120 mmHg and, once the blood had cleared, 1,000 ml of warm fixative (37°C) was introduced at the same pressure. The fixative consisted of 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.4. A further 2,000 ml of fixative was delivered at 100 mmHg and 4°C.

After perfusion, the lumbosacral spinal cord (L6–S1) and the segments containing the agar-HRP implants (T10–T12 or C2–C3) were removed and kept in the same fixative (with the omission of glutaraldehyde) for a further 4–6 hours. The blocks were washed and stored overnight in phosphate buffered saline (PBS). Transverse and sagittal sections (40 μm) of lumbosacral spinal cord were cut with a Vibratome and rinsed with several changes of PBS to ensure complete removal of excess fixative. The segments containing the implant sites were embedded in 0.3% agar and sectioned transversely with a freezing microtome at 90 μm. The C4 segment containing the control DLF lesion for the high cervical implant was postfixed for 4 days and frozen sections were cut at 90 μm.

Processing of tissue

Serial frozen sections of the implant sites were treated with 3′,3-diaminobenzidine (DAB) and hydrogen peroxide for visualization of HRP around the implant site. They were then mounted and counterstained with neutral red. Sections from the DLF lesion were treated similarly, except that they were not processed for peroxidase.

Retrogradely labelled neurons within Vibratome sections of lumbosacral cord were visualized also with DAB as the chromogen. The sections were then wet-mounted in buffer on glass slides and scanned with the aid of a light microscope. Sections containing labelled neurons were processed for CA-immunocytochemistry.

CA-containing axons were labelled with primary antisera that specifically recognise TH or DBH. The TH antiserum was raised in rabbit and its characteristics and specificity have been described by van den Pol et al. ('84). The antiserum to DBH was purchased from Eugene Tech (New Jersey), and its characteristics and specificity have been described elsewhere (Chang, '89). Vibratome sections were immersed in 10% normal donkey serum (NDS) with 0.01% sodium azide in PBS for 30 minutes to reduce nonspecific (background) staining by secondary antisera. This was followed by overnight incubation in the primary antiserum at 4°C. The antisera were diluted 1:1,000 in PBS supplemented with 1% NDS and 0.01% azide. For anti-DBH this solution was also supplemented with 0.3% Triton X-100. Although ultrastructural preservation is compromised by the addition of this agent to the incubation medium, its inclusion is essential for successful DBH-immunocytochem-

ical staining (Chang, '89; Doyle and Maxwell, '91b; Lewis and Morrison, '89). As Triton X-100 also increases antibody penetration, a number of sections to be treated with anti-TH were pretreated with 0.3% Triton X-100 for 1 hour prior to incubation in the primary antiserum.

TH and DBH were visualized in the test sections by using the avidin-biotin-peroxidase complex (ABC) technique (Hsu et al., '81). This involved incubation for 45 minutes in donkey anti-rabbit biotinylated immunoglobulin G (Amersham) diluted 1:100 at room temperature, followed by 15 min in streptavidin peroxidase complex (Amersham) diluted 1:300 at room temperature. The tissue was washed between steps in PBS and dilutions were made in PBS containing 1% NDS. The presence of peroxidase was visualized by reaction with hydrogen peroxide in the presence of DAB.

Controls

The primary antiserum was omitted from the incubation medium in control experiments. In addition, sections from the striatum were incubated with anti-TH or anti-DBH and processed identically to the lumbosacral test sections. This region possesses a dense dopaminergic network (Lindvall and Björklund, '83) and may thus be used to assess the specificity of the antisera. It would be anticipated that the anti-TH would produce a strong immunoreaction in striatal sections, while anti-DBH would produce a negative result.

Electron microscopy

After the DAB reaction, the immunostained sections were postfixed in 1% osmium tetroxide for 1 hour and dehydrated through a series of ethanol solutions. En bloc staining was performed with a 1% solution of uranyl acetate in 70% ethanol. After dehydration sections were cleared in propylene oxide, flat-embedded in Durcurpan between cellulose acetate foils, and polymerized at 60°C for 48 hours. When polymerization was complete the sections were examined with the light microscope for possible contacts between retrogradely labelled neurons and immunostained axons. A site was designated a region of contact when no space could be discerned between the varicosity and the cell at a magnification of ×1,000. The contacts were then photographed or drawn with the aid of a drawing tube.

Sections were subsequently attached to Durcurpan blocks and thin sections cut on an ultramicrotome. Ribbons of serial thin sections were collected on single-slot copper grids coated with Formvar and stained with Reynold's lead citrate for 2–3 minutes. Contacts between TH- and DBH-immunoreactive boutons and HRP-labelled neurons were examined through these series, with the electron microscope, to confirm the presence of synaptic junctions.

RESULTS

Implant sites

The implant sites from all four cats are shown in Figure 1. Drawings were constructed from a number of serial sections through the lesion and thus represent the maximum extent of the lesion. Although there was a little damage to the dorsal regions of the gray matter in some sections, the transections were otherwise confined to the dorsal columns. The lesions never extended as far as the central canal and never involved the lateral funiculi. These reconstructions confirmed that the thoracic implants (T10–T12) were bilateral (Fig. 1A–C) and that the cervical implant (C2–C3) was confined to the left hand side of the cord (Fig. 1D). The reaction product of HRP from the pellet was most intense in the region of the implant, but some reaction product was usually observed surrounding it in the gray matter and the DLF. The control lesion, caudal to the cervical implant, was accurately made in the DLF at C4 (Fig. 1E).

Retrograde labelling

The distributions of retrogradely labelled neurons were comparable to those reported in previous studies (Bennett et al., '83; Enevoldson and Gordon, '89a; Giesler et al., '84; Rustioni, '76; Rustioni and Kaufman, '77), but differences may exist between labelling produced by implants at the two regions of the spinal cord. Bilateral HRP implants in the thoracic cord produced large numbers of labelled neurons within laminae III, IV, and medial V. A much smaller second concentration of labelled cells was found in the medial portions of laminae VI and VII and an occasional cell was observed in lamina I. These cells were found in roughly equal numbers on both sides of the cord. The unilateral cervical implant produced a much more restricted distribution of labelling, although a much smaller population of neurons was sampled. With the exception of a single neuron in lamina VII, all retrograde labelling was confined to laminae III–V.

The retrogradely transported HRP granules were present in the perikarya and proximal dendrites of labelled neurons (Figs. 3, 4). Labelled primary and secondary dendrites were observed routinely but tertiary dendrites were rare. Axons were sometimes labelled, but their collaterals were not apparent. It was noted that cells labelled from the cervical implant (Fig. 4) usually contained less HRP reaction product than those labelled from the more caudal implant (Fig. 3).

Light microscopic observations of immunoreactive varicosities and postsynaptic dorsal column neurons

Large numbers of axons immunoreactive for TH and DBH were observed throughout the lumbosacral spinal dorsal horn, but in accordance with our previous studies (Doyle and Maxwell, '91a,b), they were particularly abundant within the superficial layers (laminae I and II) and medial lamina IV. It was noted that the density of immunostaining produced with the DBH antiserum was greater than that observed with the anti-TH, even when comparing TH-labelled material treated with Triton X-100. This was not due to the Triton X-100 incubation period being longer for DBH-immunocytochemistry, since reducing it to 1 hour did not alter the immunoreaction.

All retrogradely labelled neurons examined for immunoreactive contacts were within laminae III–V. Sixty-seven out of 107 cells (62.5%) labelled following the thoracic implants possessed DBH-immunoreactive terminals closely apposed to their perikarya and/or proximal dendrites (mean number of contacts per cell = 4.4, range = 1–16). Examples are shown in Figure 3E,F. Twenty-three out of 39 cells (59.0%) labelled from the cervical implant received contacts from DBH-immunostained axons (mean number of contacts per cell = 4.9, range = 1–22). Examples are shown in Figure 4. One hundred ninety-seven cells labelled from the thoracic implants were examined for contacts from TH-immunoreactive axons. Analysis of tissue that had been treated with Triton X-100 revealed that 34% of neurons had

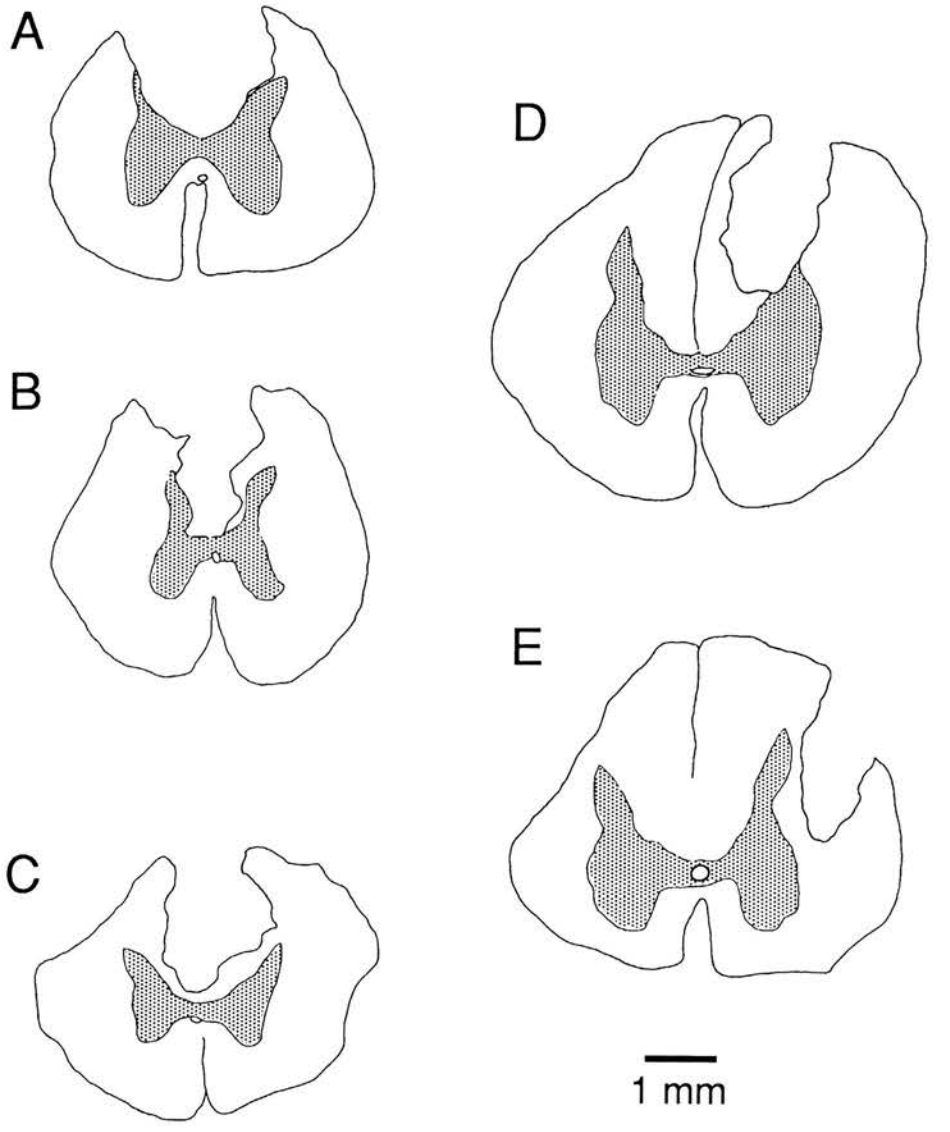

Fig. 1. Drawings of thoracic (**A–C**) and cervical (**D,E**) spinal cord, illustrating the area of destruction associated with the dorsal column implant sites (A–D) and the cervical dorsolateral funiculus (DLF) lesion (E). The drawings show the maximum extent of each lesion.

terminals apposed to their somata and/or proximal dendrites (mean number of contacts per cell = 3.2, range = 1–13). Examples are shown in Figure 3A–D (see also tissue prepared for combined light and electron microscopy: Figs. 5, 6). The apparent lower innervation from axons containing TH is a consequence of the weaker immunoreaction observed with this antiserum.

Fibres immunoreactive for TH and DBH formed both terminal and en passant axonal swellings, and individual collaterals gave rise to both single (Fig. 3A) and multiple (Figs. 3, 4) contacts. Frequently, axons could be seen to follow closely the curvature of dendrites or somata (Figs. 3C–F, 4). Many retrogradely labelled neurons were innervated by several immunostained axons (Figs. 3, 4A). In accordance with our previous findings (Doyle and Maxwell, '91a) at least two different morphological types of axon can be distinguished by the dimensions of the varicosities that occur along the axonal strands. Both types of axon appear to innervate PSDC cells. The more common type of axon had both large and small varicosities intermingled along its length (Figs. 3A–D, 4), while the second, less common type was characterized by small varicosities that were only slightly larger than their intervaricose axons (Fig. 3E,F).

The laminar locations of 44 labelled cells, observed in transverse sections, receiving contacts from TH- or DBH-immunostained contacts are shown in Figure 2. Approximately 70% of the cells are located in the medial half of laminae III–V, where most of the CA-containing fibres are found.

Controls

Sections that had been incubated in a medium from which the primary antiserum had been omitted displayed no immunolabelling. Sections from the striatum that had been treated with the TH antiserum displayed an intense

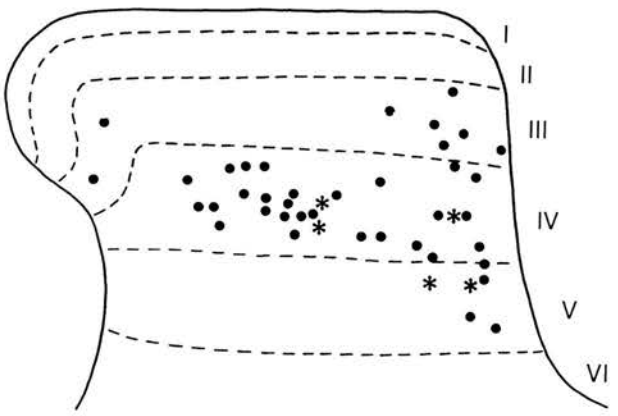

Fig. 2. The laminar locations of 44 retrogradely labelled neurons, found within laminae III–V, which received contacts from catecholamine (CA)-containing axons. The position of each cell body has been placed on a standard dorsal horn according to its relative position both dorsoventrally and mediolaterally in its own dorsal horn. Note that most of the innervated cells (70%) were found within the medial half of the dorsal horn. Five labelled cells receiving contacts from CA-containing boutons that were confirmed ultrastructurally to be synaptic associations are indicated (asterisks).

immunoreaction, which was absent from the sections incubated with anti-DBH.

Electron microscopic observations of immunoreactive varicosities and postsynaptic dorsal column neurons

Ultrastructural examination of retrogradely labelled neurons demonstrated that they contained HRP reaction product that was sequestered within membrane-bound granules (Figs. 5, 6). Such electron-dense bodies are typical of retrogradely transported HRP (La Vail and La Vail, '74). In addition to membrane-bound HRP some neurons contained diffuse reaction product that was low in intensity and usually associated with membranous structures.

Varicosities observed with the light microscope were revealed by electron microscopic analysis to be synaptic boutons. The appearance of these boutons was comparable with those reported in our previous studies (Doyle and Maxwell, '91a,b). They contained large numbers of small pleomorphic (round to oval) agranular vesicles together with several dense core vesicles. By using correlated light and electron microscopy, we were able to confirm that 12 CA-containing boutons formed synaptic specializations with the dendrites (Fig. 6) and somata (Fig. 5) of labelled cells. These synapses could be followed for up to 9 serial sections.

On 8 occasions, axonal swellings that formed apparent contacts at the light microscopic level were found not to make synaptic associations with retrogradely labelled structures when viewed with the electron microscope. Seven of these structures synapsed upon an adjacent, unlabelled structure. The remaining bouton did not form an identifiable synaptic junction and lay closely apposed to the soma of a retrogradely labelled cell.

DISCUSSION

Identification of PSDC units

The electrophysiological studies of Angaut-Petit ('75a) have shown that 87% of ascending second-order axons in the thoracic dorsal columns project to the first cervical segment. At C2–C3, it is probable that all dorsal column axons originating in the lumbar cord belong to this pathway.

Anatomical studies in the cat have shown differences in the laminar distribution of labelled cells after HRP applications to the thoracic dorsal columns and the dorsal column nuclei (DCN). Rustioni and Kaufman ('77) and Enevoldson and Gordon ('89a) found that lumbar retrograde labelling was almost entirely confined to laminae III–V after HRP was applied to the DCN. The cells labelled by Rustioni and Kaufman ('77) also included the small population of spino-DCN cells ascending the DLF (Enevoldson and Gordon, '89b), since these authors made no lesion in the DLF. When HRP was placed into the dorsal columns at T9–T12, an additional cluster of cells was revealed in medial laminae VI–VII, as well as a few cells in lamina I (Bennett et al., '83). We also observed this pattern of retrograde labelling, and concluded that the cells labelled in laminae I, VI, and VII are long propiospinal neurons and correspond to the small population (13%) of non-PSDC, second-order cells observed ascending the thoracic dorsal columns by Angaut-Petit ('75a). On this basis, we confined our examination to labelled cells within laminae III–V, since these neurons probably project to the DCN. In this context, it may be significant that Rustioni and his colleagues only found retrograde labelling within laminae I and VI–VII when their medullary HRP injections were not restricted to the DCN (Rustioni, '76; Rustioni and Kaufman, '77). However, despite these precautions, we cannot eliminate the possibility that a few of the cells examined may have been propiospinal.

The agar-HRP implantation technique employed in this study depends upon uptake of HRP by the severed ends of axons but not by axons of passage. Considerable evidence supporting this claim has been presented in a number of publications (Bennett et al., '83; Enevoldson and Gordon, '89a; Enevoldson et al., '84). Enevoldson and Gordon ('89a) and Bennett et al. ('83) performed control experiments involving lesions of the DLF caudal to the site of the implant. Both studies revealed that there was no alteration in retrograde labelling as a result of the additional lesion and concluded that HRP had not been taken up by axons in the DLF. In addition, Enevoldson et al. ('84) found a critical relationship between the number of cells labelled and the extent of the dorsal column lesion. Examination of our lesion sites indicated that there was some spread of HRP into the DLF. However, as no damage of the DLF was observed in any of the sections surrounding the lesion site, it is unlikely that any labelling of axons in this tract occurred.

It is also possible that spread of HRP into the gray matter surrounding the lesion site could result in labelling of propiospinal neurons with axons terminating in this region. This is unlikely, however, since Bennett et al. ('83) found that lesioning the dorsal columns caudal to the implant resulted in a total absence of retrograde labelling.

Innervation of postsynaptic dorsal column neurons by catecholamine-containing axons

The combined light and electron microscopic data presented in this report indicate that lumbosacral PSDC neurons in the cat are innervated by CA-containing axons originating in the brain. Light microscopic observations suggest that about 60% of cells possess CA-containing terminals closely apposed to their somata and/or proximal

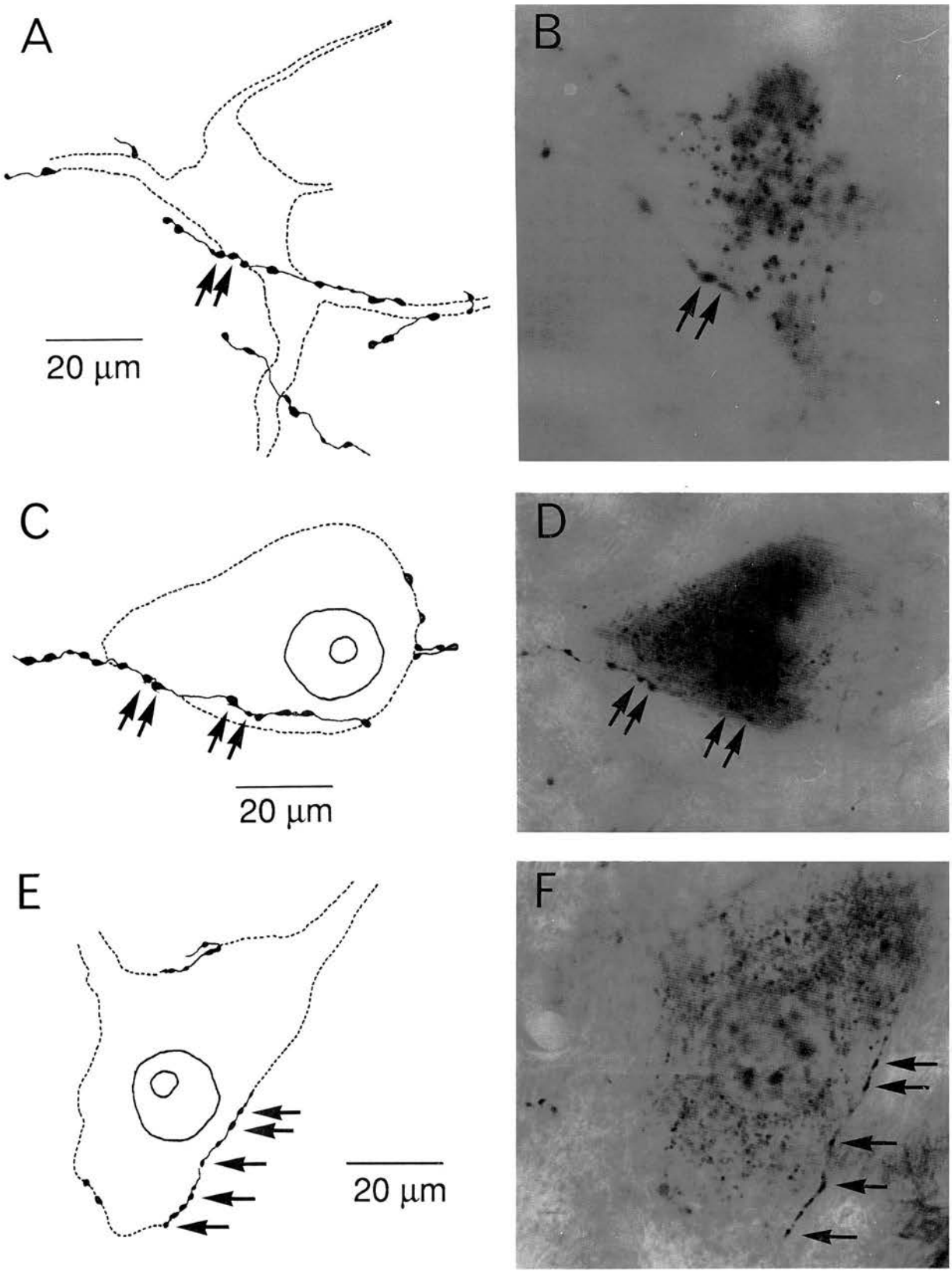

Fig. 3. **A, C, E:** Drawings of 3 retrogradely labelled postsynaptic dorsal column (PSDC) neurons observed after horseradish peroxidase (HRP) implantation into thoracic spinal cord. The arrows point to tyrosine hydroxylase (TH)-immunoreactive (A, C) or dopamine-β-hydroxylase (DBH)-immunoreactive (E) varicosities, which are also shown in the corresponding light micrographs (**B, D, F**).

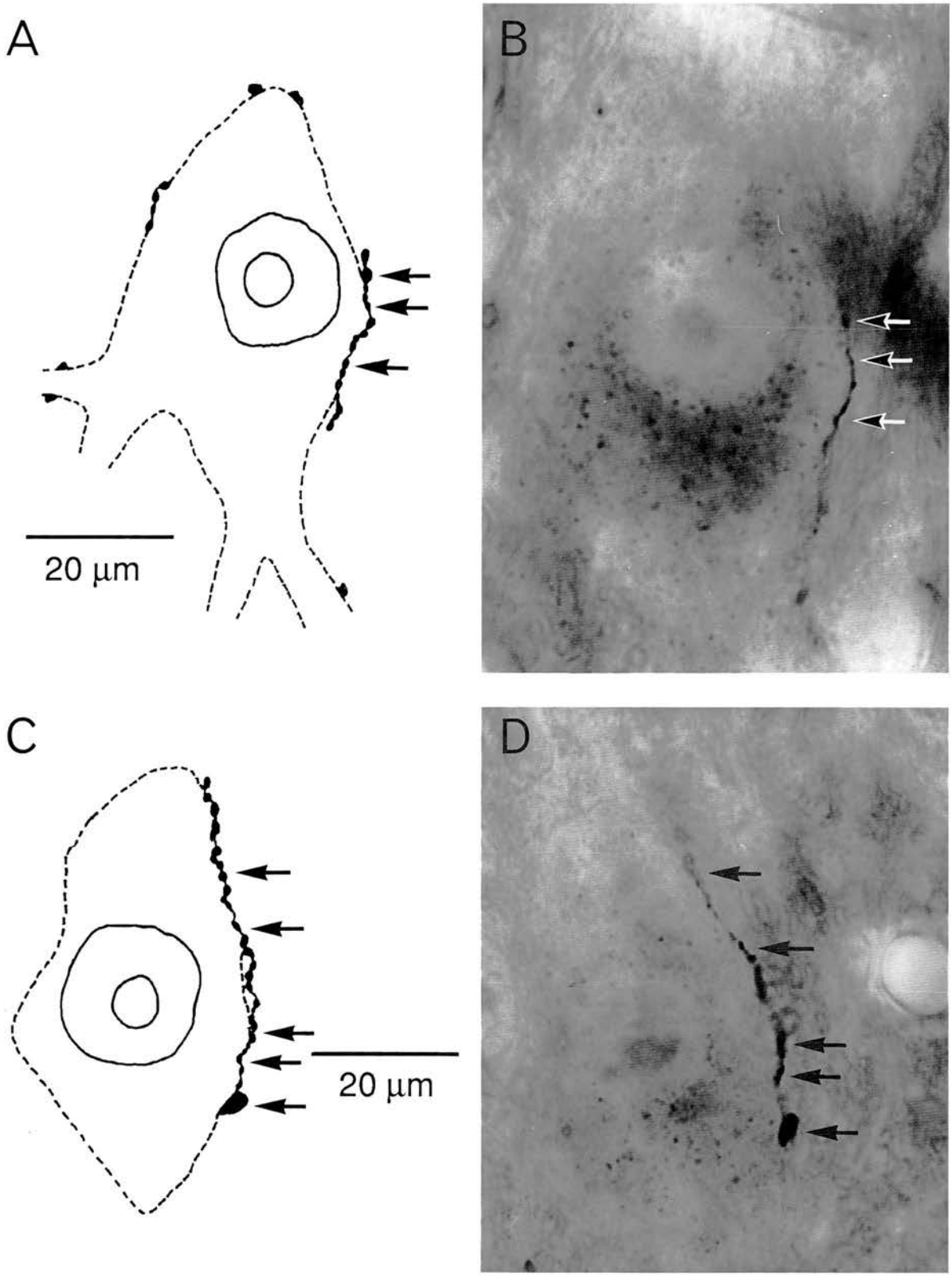

Fig. 4. **A,C:** Drawings of 2 retrogradely labelled PSDC neurons, observed after implantation of HRP into cervical (C2–C3) spinal cord, that were innervated by axons immunoreactive for DBH. The arrows point to varicosities, which are also shown in the corresponding light micrographs (**B,D**).

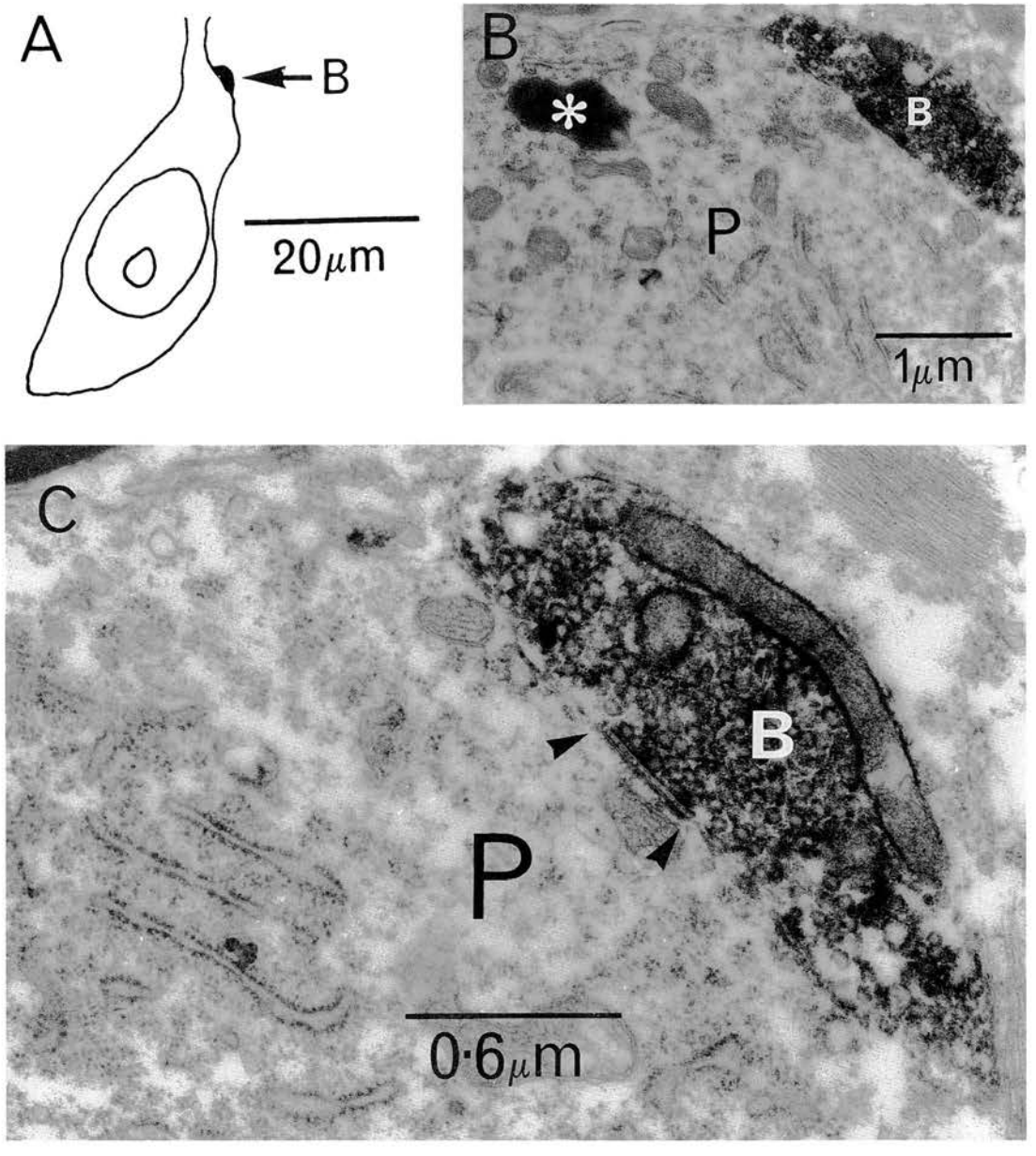

Fig. 5. Correlated light and electron microscopic analysis of a PSDC neuron from lamina IV that received a TH-immunoreactive axosomatic contact. **A:** Drawing of the retrogradely labelled neuron and immunostained bouton (B). **B:** Electron micrograph of the neuron. The bouton (B) directly apposes the perikaryon (P), which contains a granule of retrogradely transported HRP (asterisk). **C:** At higher magnification the synaptic junction formed by this bouton is revealed (between the arrowheads).

dendrites, with some cells receiving up to 22 contacts. However, estimates of frequency of contacts are likely to be limited in this study for several reasons. 1) Our previous ultrastructural studies (Doyle and Maxwell, '91a,b) indicated that the majority of synaptic contacts formed by boutons of CA-containing fibres are on fairly small (less than 2 μm diameter) dendritic shafts, which are probably distal dendrites. As the retrograde labelling technique employed in this study resulted in the labelling of somata and proximal dendrites only, it is probable that the majority of contacts between CA-containing fibres and PSDC neurons would not be detected. 2) The presence of glutaraldehyde in the fixative reduces immunocytochemical staining by denaturing the target antigen. Hence it is unlikely that all of the catecholaminergic innervation to the dorsal horn was actually labelled. In addition, the high concentrations of paraformaldehyde in the fixative, along with using a relatively insensitive chromogen (DAB), would also reduce the number of labelled cells.

Although light microscopy revealed that over 60% of PSDC neurons receive contacts from CA-containing axons, its resolution (0.2 μm) is not sufficient to exclude the possibility that structures in apparent apposition are actually separated by an intervening neuronal or glial process, or that a varicosity in contact with a labelled cell is actually synapsing on some adjacent unlabelled structure and not

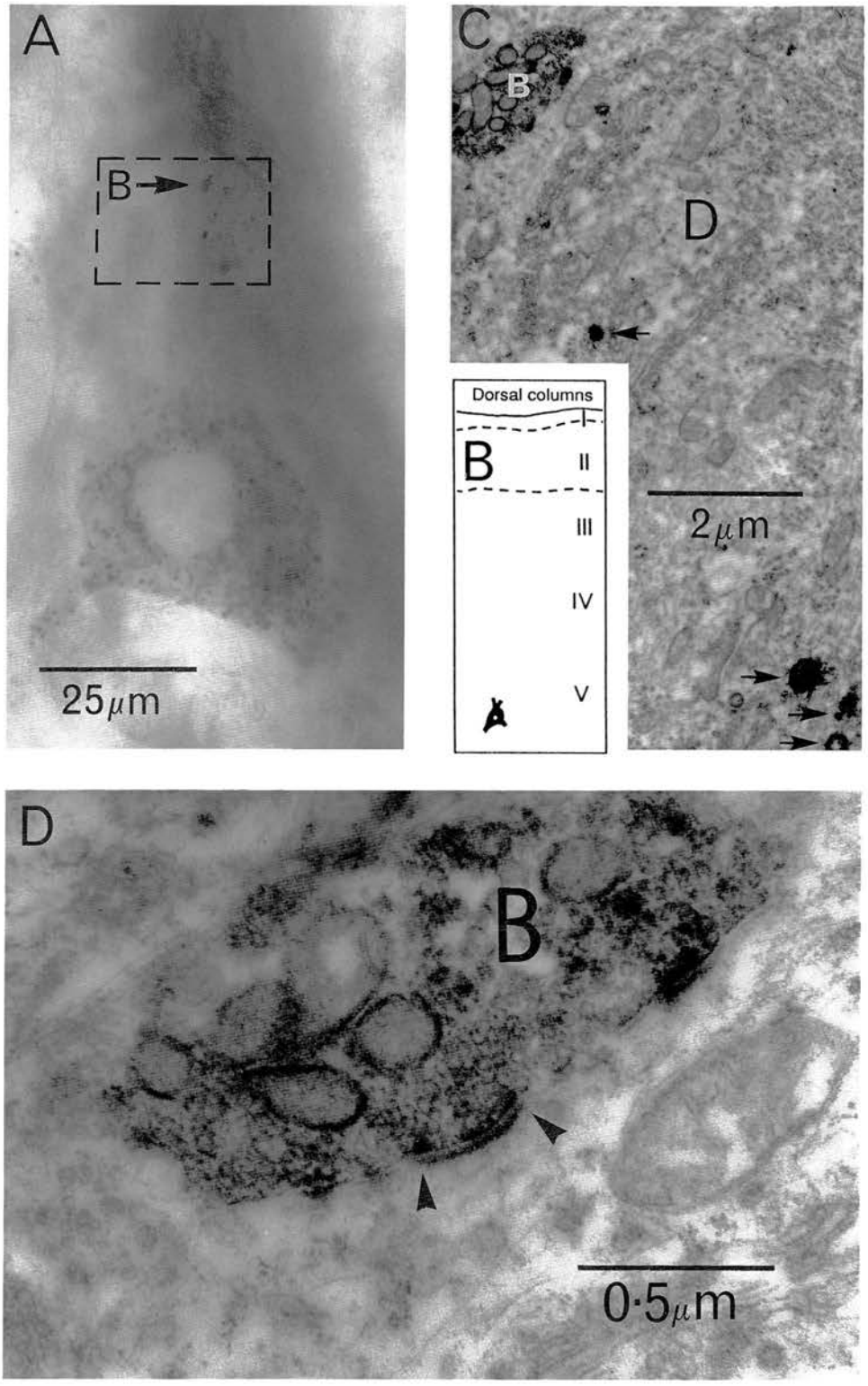

Fig. 6. Correlated light and electron microscopic analysis of a PSDC neuron receiving a TH-immunoreactive axodendritic contact. **A:** Light micrograph of the retrogradely labelled neuron and the immunostained bouton (B) contacting it. Note the presence of HRP granules within the cell body and proximal dendrites. The area enclosed by the **box** is shown at a higher magnification in electron micrograph **C.** Note the granules of retrograde HRP (arrows) within the dendrite (D) and the contact from the immunostained bouton (B). **B** shows the location of the cell body in lamina V. At higher magnification (**D**) the synaptic junction formed by this bouton is revealed (between the arrowheads).

the cell itself. Correlated ultrastructural analysis, however, verified that many contacts between CA-containing fibres and labelled neurons (12/20) were regions of synaptic association. These findings suggest that many of the contacts seen with the light microscope alone were regions of synaptic interaction. However, 8 axonal swellings that appeared to contact labelled cells were found not to form synapses with the cells when viewed ultrastructurally and therefore it cannot be assumed that all contacts observed with the light microscope are synaptic connections. Seven of these boutons synapsed on an adjacent, unlabelled dendrite and one bouton did not form a synaptic junction. Descending CA-containing boutons that do not form identifiable synaptic junctions are rare (Doyle and Maxwell, '91a,b), but a few examples have recently been found (unpublished data).

Functional considerations

Previous studies have suggested that the spinal CAergic system plays an important role in controlling the transmission of nociceptive information to supraspinal sites. For instance, behavioural studies have shown that intrathecal administration of sympathomimetic drugs inhibits reflexes evoked by noxious cutaneous stimuli (Jensen and Smith, '83; Kuraishi et al., '79; Reddy et al., '80; Yaksh and Reddy, '81). In addition, electrophysiological recordings from unidentified dorsal horn neurons (Belcher et al., '78; Davies and Quinlan, '85), spinocervical tract neurons (Fleetwood-Walker et al., '85, '88), PSDC neurons (Fleetwood-Walker et al., '85), and neurons of the spinothalamic and spinomesencephalic pathways (Fleetwood-Walker et al., '88) have demonstrated that excitation induced by noxious cutaneous stimulation may be inhibited by CAs. In these studies nociceptive responses were depressed to a much greater extent than responses to innocuous stimuli, and the authors proposed that the descending CA pathways act selectively to inhibit nociceptive transmission. However, other groups have found that noradrenaline attenuates the responses to noxious and non-noxious stimuli with equal potency (Howe and Zieglgänsberger, '87; Satoh et al., '79). Hence, the precise effects of catecholamines in the spinal cord are at present uncertain. In this study we have shown that spinal neurons that project to the dorsal column nuclei via the dorsal columns (PSDC neurons) are innervated by CA-containing axons. Electrophysiological studies have demonstrated that most of these neurons (62– 77%) respond to a wide range of stimuli including hair movement, noxious pinch, and heat, and only a very small minority are nociceptive specific (about 3%) (Angaut-Petit, '75b; Brown et al., '83; Giesler and Cliffer, '85). Hence, the predicted effect of the CAergic axons innervating most PSDC neurons would be to suppress excitation generated by both light-tactile and nociceptive stimuli. Headley et al. ('78) recorded from multireceptive neurons in laminae III and IV and compared the effect of ionophoresing noradrenaline in the substantia gelatinosa with ejection in the vicinity of the neuron being tested. Although ionophoresis in the substantia gelatinosa produced a specific inhibition of nociception, ionophoresis close to the cell body resulted in a much less selective action. This nonspecific inhibition by noradrenaline of laminae III–IV neurons is therefore compatible with the suggestion that the direct action of CA-containing axons on PSDC neurons would be a general suppression of excitation and not selective inhibition of nociceptive input.

Results from brainstem stimulation experiments suggest that CAergic axons in the dorsal horn may operate via two separate mechanisms. Stimulation of the nucleus locus coeruleus (Hodge et al., '83; Mokha et al., '85; Zhao and Duggan, '88) and the dopaminergic A11 cell group (Fleetwood-Walker et al., '88) has been shown to depress nociceptive responses with far greater potency than responses to innocuous stimuli, while activation of the subcoeruleus-parabrachial nuclei (Girardot et al., '87) and Kölliker-Fuse nucleus (Zhao and Duggan, '88) produces a nonselective inhibition of sensory transmission. In the cat, the Kölliker-Fuse nucleus is the primary source of CA in the lumbar spinal cord (Stevens et al., '82). Hence, most of the terminals examined in the present study will have originated from this nucleus. In this context, our present findings provide a good morphological correlate for the nonselective inhibition of nociceptive and non-nociceptive responses that Kölliker-Fuse nucleus stimulation evokes in the cat (Zhao and Duggan, '88).

CONCLUSIONS

Our analysis of postsynaptic dorsal column neurons in laminae III–V of the cat spinal dorsal horn indicates that over 60% of cells receive contacts from catecholamine-containing terminals upon their somata and proximal dendritic arbors. Ultrastructural analysis confirmed that many of these contacts were regions of synaptic specialization. These observations suggest that catecholamines regulate sensory transmission through this spinomedullary pathway by a direct postsynaptic action upon its cells of origin. Such an action would be predicted to suppress transmission generally through this system.

ACKNOWLEDGMENTS

Special thanks are due to Heather Anderson for her skilled technical assistance. We are also grateful to Dr. Cali Ingham for critically reviewing the manuscript. The study was funded by the Medical Research Council.

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